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SCIENTIFIC RATIONALE FOR THE SELECTION OF TOXICITY TESTING METHODS
II. TERATOLOGY, IMMUNOTOXICOLOGY, AND INHALATION TOXICOLOGY

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ABSTRACT

This document is the second of a two-part literature analysis of parameters associated with the various toxicity testing methods (test animal selection, pathology, etc.). Acute, subchronic, chronic, and carcinogenic testing methods are covered in ORNL/EIS-151. Testing methods for developmental toxicity, immunotoxicology, and inhalation toxicology and research needs associated with these areas are covered in this volume, ORNL-6094. These reports were prepared for the purpose of assisting and supporting the U.S. Environmental Protection Agency in its efforts to develop guidelines for more efficient and economical testing procedures.

1. EXECUTIVE SUMMARY

1.1 TERATOGENICITY

Proper attention to the design of a teratogenicity experiment is essential. Parameters such as the number and level of doses, the number of species, the number of test animals per dose group, and the time during pregnancy for the administration of the test agent are important considerations.

At least three dose levels should be tested; the low dose should permit normal embryonic development, the intermediate dose(s) should produce malformed offspring if the test chemical is teratogenic, and the high dose should be maternally toxic or produce embryotoxicity or fetotoxicity. At least two species of test animals with a sufficient number of animals per dose group to permit meaningful statistical evaluation of the results should be used. Current guidelines recommend the use of at least 20 pregnant animals of a rodent species and 12 pregnant rabbits. Generally, the test agent is administered during the period of organogenesis because this is considered to be the developmental stage most sensitive to the action of teratogenic chemicals. The period of prenatal development that slightly overlaps organogenesis but extends primarily into the fetal period is known as histogenesis. Although teratogenic agents that come into contact with the developing fetus during this time can cause minor structural deviations, the abnormalities that are more likely to occur during the fetal period are those involving growth or functional aspects of development.

The term behavioral teratogenesis is used to describe the branch of teratology devoted to studying behavioral modifications that result from prenatally administered agents. In addition to the histogenesis period, administration of chemicals during organogenesis or pregestationally has resulted in behavioral alterations in offspring.

Although many different animal species have been used as test animals in teratogenicity testing experiments, no ideal species has been identified. The rat and mouse are the most commonly used rodent species, and the rabbit is the most commonly used nonrodent species. The nonhuman primate has received considerable attention as a test animal because of its anatomical and physiological similarity to man; however, the cost associated with the use of nonhuman primates will likely restrict their use to special situations (e.g., testing a drug designed for use during pregnancy).

In recent years alternative approaches to the standard methods of testing agents for teratogenicity have been investigated, primarily due to economic and practical considerations. These include an in vivo method where determinations of teratogenic potential are made on the basis of litter size and weight and several in vitro methods, such as whole mammalian embryo culture, a *Hydra attenuata* system, and a frog embryo assay.

Possible areas for future research include further investigation of postnatal behavioral changes as they relate to pregestational and gestational chemical exposure, further research directed toward standardization of the methodology for screening chemicals for behavioral teratogenicity, and determination of an acceptable battery of tests for in vitro screening of compounds for teratogenicity.

1.2 IMMUNOTOXICOLOGY

Investigations into the toxic effects of industrial and environmental chemicals have revealed that some of these chemicals have distinct, and usually very specific, effects on the immune system. This complex organization of organs and cells plays a critical role in the protection of man against infection and neoplastic diseases; thus dysfunction of the system can have serious health effects. These issues are the basis for the evolution of the field of immunotoxicology (the study of immunologic alterations caused by industrial and environmental chemicals), and they have stimulated interest in test methods originally designed for use in immunopharmacology (to assess the therapeutic potential of immunosuppressant or immunopotentiating drugs). Several toxicology groups have suggested the use of immunotoxicology assays as an adjunct to toxicology studies.

The protective responses of the body against foreign entities (antigens) are carried out by the immune system, which is composed of cells and organs of the lymphoreticular system. Immune responses may be specific or nonspecific.

The specific immune response is characterized by the highly specific recognition of (and response to) antigen by lymphocytes and by the induction of immunological memory. Specific immunity includes cell-mediated immunity (an expression of the activities of T-lymphocytes), humoral immunity (an expression of the activities of B-lymphocytes), and immunological tolerance.

When a chemical compound is suspected of altering the immune response, both the cellular and humoral responses must be tested. Tests of cell-mediated immunity usually measure T-cell activities, either in vivo or in vitro. Basic in vivo procedures for evaluating the cell-mediated immune response include tests of delayed hypersensitivity, allograft rejection, and graft vs host reactions. These are well-established, valuable procedures but are generally cumbersome and time consuming, requiring large numbers of animals. Newer tests have been developed, some utilizing isotopes, that are more efficient, quantitative, and sensitive. Some of these, such as the radiometric ear test and the footpad assay, have been recommended for inclusion in toxicity screening protocols.

In vitro tests of cell-mediated immunity are versatile and convenient methods for the large-scale screening of chemicals. Tests recommended for screening protocols include lymphocyte transformation by mitogens, mixed lymphocyte cultures, and assays for the migration inhibiton factor. For these assays, the test substance is generally administered to the test animal, appropriate cells are removed, and immunocompetence of these cells is measured in vitro. In some assays, however, the test chemical can be added to the test system in vitro, thus bypassing the animal treatment step.

Tests of humoral immunity measure, in some cases, both T- and B-cell functions (T-cell dependent humoral response) and, in others, only B-cell functions (T-cell independent humoral response), depending on the antigen used. Humoral immunity is generally evaluated on the basis of circulating antibody or local production of antibody, the identification and quantitation of the various immunoglobulins, and the colony-forming and blastogenic capabilities of B-cells. Several of these tests have been suggested for use in toxicity screening protocols. These include assays for circulating antibody, the classic hemagglutination and hemolysin titrations, and more modern techniques, such as single radial immunodiffusion (SRID) assay and the enzyme-linked immunosorbent assay (ELISA). Tests designed to detect the local production of antibody include the plaque-forming assay (localized hemolysis in gel), immunofluorescence techniques, and erythrocyte-antibody-complement (EAC) rosette assay.

The nonspecific immune response does not involve the recognition of antigen or the mounting of an immune response but, instead, involves the generalized activities of phagocytic cells, lysozymes, the interferon system, the complement system, and the kinin system. Tests for macrophage function, hormone and complement activities, host resistance to infection, resistance to tumor challenge, and endotoxin hypersensitivity are typical procedures used to assess this system.

Another facet of immunology test procedures, also important in assessing the toxic properties of industrial/environmental chemicals, are tests for allergencity, which, in contrast to immunotoxicity procedures that measure immunocompetence, are designed to measure the sensitizing potential of a chemical. Chemical allergens found in industrial environments, thought to be highly protein-reactive chemicals, usually affect a small subset of workers who are hypersusceptible to the low-dose exposure legally permitted in chemical plants. Sensitization can occur via dermal or respiratory routes, and, in either case, the resulting allergic reaction can have serious ramifications for the worker involved. Predictive tests in animals, usually guinea pigs, have been designed to assess the allergenic potential of chemicals. Skin procedures include the Draize test, Freund's complete adjuvant test, the guinea pig maximization test, the "split adjuvant" test, and the open cutaneous test. Respiratory sensitization in animals, although difficult to attain, has been demonstrated in limited studies.

If assays to evaluate the immunotoxic effects and allergic potential of chemicals are to become incorporated in routine toxicity test protocols, the selection of the proper combination of tests that would be most efficient, economical, and informative becomes an important consideration. Experts in the field of immunotoxicology have made their independent recommendations for the types of immunology procedures that should be included in routine toxicity studies and have designed tier-testing schemes to demonstrate how this can be accomplished.

1.3 INHALATION TOXICOLOGY

1.3.1 General Test Design

Toxicity tests designed to evaluate effects from inhalation exposure involve many of the same variables as tests utilizing other exposure routes. The selection of test species should be based on the anatomical and physiological similarity of the respiratory tracts of humans and the test species, as well as metabolic similarities and economic/practicality considerations. Larger animals (e.g., horses, monkeys) are generally more similar to humans, but practical restraints favor the use of smaller animals (e.g., rats).

Test durations for inhalation studies include acute (one exposure with 14 days of observation), subchronic (repeated exposures for 14, 28, or 90 days), and chronic (repeated exposures for 1 to 3 years). Due to the large costs involved in a chronic study, these should be undertaken only when preliminary studies indicate the need for an evaluation of lifetime effects. Exposure to the test agent can be intermittent (6 to 8 h per day, 5 days per week), based on occupational exposure, or continuous (22 to 24 h per day, 7 days per week) to simulate environmental exposure. Intermittent exposure allows recovery from the test agent (which can affect toxicity) but is generally simpler to maintain and operate.

Inhalation studies with repeated exposures should include at least three dose levels and suitable controls. The determination of the actual dose delivered to the test animal is a major difficulty in inhalation studies. The common method is to calculate the dose based on the concentration (C) in the test chamber multiplied by the length of exposure (T). However, this indicates only the maximum possible dose because not all of this quantity is inhaled by the animal or, once inhaled, reaches the lungs. Dosimetric formulas have been developed that consider the volume intake of the lungs and the percentage of the agent retained in the lungs. Unfortunately, these variables are difficult to determine under actual use conditions, and the $C \times T$ estimation of dose is still widely used.

The evaluation of toxic effects resulting from inhalation exposure includes many of the procedures used in other toxicity studies as well as procedures specific for inhalation-caused effects. Clinical observations of the animals should be made daily, before, during, and after exposure. These include signs of irritation, behavioral changes, and changes in bodily functions. Biochemical and hematological tests should be performed on blood samples (taken before and after exposure) to aid in evaluating systemic effects. Biochemical tests for evaluating lung damage are currently being modified from similar tests used to evaluate hepatotoxicity. Pathological techniques are the most useful evaluations for assessing toxic effects from inhalation exposure. A thorough gross examination of all test animals is necessary with special attention given to the respiratory system. Microscopic evaluations should be performed on animals from the high dose and control groups as well as any lesion-bearing animals found in the gross examination. The organs to be examined should cover any potential targets for systemic toxicity. Respiratory tissues should be examined in detail, using special techniques such as histochemistry, when indicated. Respiratory function tests are techniques for evaluating inhalation exposure effects that require special equipment and training. They can quantify changes at low-dose levels and can produce dose-response data. However, they are not conclusive by themselves and should be correlated with other toxicity evaluations.

1.3.2 Exposure Chamber Design

The design of the inhalation exposure chamber is an important consideration in an inhalation study. It can be operated in a static mode, where a predetermined dose is introduced into a closed chamber, or in a dynamic mode, where a continuous flow of air and test agent is vented through the chamber. Static exposures are limited by problems associated with the finite volume of air used without replacement and are generally most useful for short-term exposures or when the test agent is available only in limited quantities. Dynamic exposures are used for the majority of inhalation tests and require accurate calculations of airflow and test agent feed rates to achieve the desired doses. The chambers are most frequently designed to house the whole animal within the exposure area, particularly for long-term studies. Nose- and head-only systems can be used when dermal or accidental oral exposure must be avoided. These chamber designs require special equipment to restrain the animals and seals to prevent the loss of test agent.

The shape of most large, dynamic, whole-body exposure chambers is based on a cubical or hexagonal design. Pyramidal or conical additions to the top and bottom of the chamber increase the distribution efficiency of air within the chamber, making the design closer in operational characteristics to the most efficient shape, the sphere. The most widely used chambers of this design are the Rochester (hexagonal) and New York University (cubical) designs. For carcinogenesis studies, more isolated chambers or exposure rooms are often used because of the long duration of the tests and the need to limit exposure to the test compound. Small-scale exposure chambers designed for pilot or acute studies occur in more variations including bell jars, Lucite cylinders, and scaled-down versions of larger units. Flexibility is important in these designs since the unit will usually be used for many types of chemical exposures.

Chamber size is governed primarily by air distribution and animal volume considerations. The maximum animal volume should rarely be more than 5% of the total chamber volume, and chambers of 1 to 8 cubic meters usually provide the best air distribution.

The chambers are usually constructed of smooth, nonabsorbent materials that are resistant to a wide range of chemicals. Stainless steel walls and glass or Plexiglass® observation ports and doors are the most common materials. Rubber or plastic seals are necessary to prevent air loss and should be resistant to the test chemical. Animal cages are generally made of stainless steel mesh without any solid surfaces. Their arrangement should facilitate exposure with rotation of the individual positions for long-term studies.

Airflow systems for the chamber should provide units to condition, filter, meter, and control the flow of the air, test agent, and their mixture. The rate of airflow is determined by the animal loading and the test agent feed rates. Various meters and valve arrangements are used to provide the necessary control, with valves located downstream from filter units to prevent clogging. An air pump is usually located in the exhaust line to provide the negative operational pressure (0.1 to 0.5 in. of water) for the chamber. Air enters the chamber through the top pyramid and usually exits through an arm of a Y-joint at the bottom pyramid. The air needs to be conditioned to control temperature and humidity within an acceptable range (75 to 82°F and <55% humidity). Automatic control systems for this are available. Filter banks should be placed in the main supply line and in the chamber and main exhaust lines. Usually several types of filters are included in each bank to ensure satisfactory removal of contaminants.

During operation of the chamber, samples must be taken to monitor the exposure conditions, including temperature, humidity, and test agent concentrations (also particle sizes for aerosol atmospheres). Sampling probes should monitor all areas of the chamber distribution with added emphasis on conditions at the breathing zone of the animals. Sampling and monitoring techniques for vapor and gas atmospheres usually utilize filters with standard analytic techniques. Aerosol atmospheres are more difficult to sample, and a whole array of samplers have been developed for this, including elutriators, cascade impactors, centrifugal force samplers, precipitators, and optical monitors. These usually include some means for separating samples into size classes to determine particle size, number, and concentration.

To maintain sanitary conditions within the chamber, techniques and equipment have been developed to remove animal wastes. Usually a permanent spray ring is used to wash down the chamber walls with an appropriate cleaning solution. Cleaning procedures must be followed that prevent cross contamination of different chambers and that protect the animal technicians.

Occasional use of specialized chambers or equipment may be necessary. Examples of such equipment include chambers that operate under reduced or increased pressures and apparatus to evaluate effects of animal activity during exposure to test agents.

1.3.3 Generation of Test Atmospheres

Inhalation testing requires that the test agent be put into an atmospheric form for exposure. Atmospheres of gas or vapor test agents are relatively simple to generate, compared to substances that must be in an aerosol form.

Gases and vapors can be classified as irritants or asphyxiants. One common method for generating gas and vapor atmospheres is to store the chemical in high pressure cylinders. For batch introduction, air can be mixed into the cylinder, while for dynamic systems the gas can be released into the airstream. Porous diffusion plugs or containers can also be used to release gases. If the substance is not volatile or chemically stable enough to store in cylinders, it can be vaporized through controlled heating or counter-current techniques.

The generation of aerosols must include techniques for many types of aerosols including dusts, fumes, smoke, mists, and fogs using solid or fluid feed materials. Aerosols occur in monodisperse forms where the particles are all within a narrow size range or polydisperse forms that include a wide range of particle sizes. Monodisperse aerosols are more useful for studying particle size effects since they simplify the experimental conditions, but polydisperse aerosols are more typical of actual exposure conditions. The division of the parent material into particles or droplets of a small enough size to remain airborne intensifies the chemical and physical activities of the test agent because of the increased surface area and total space occupied.

The generation of aerosols from solid test substances has been performed using loose and compacted forms of the particles. Loose particles must be kept in suspension by vibrating, rotating, or stirring techniques usually combined with an aspirator to distribute the particles. Such techniques are used more often for handling fibrous dusts or to produce polydisperse aerosols. The most common technique for generating dust aerosols is to use a compacted plug of powder and then scrape off particles to redistribute. The Wright dust feed generator is a typical apparatus of this design and uses a horizontal, rotating scraper blade and a packed plug of powder in a brass cup. By controlling the humidity of the distributing air stream, plus the packing density and particle sizes of the plug, there should be no problem with redistributing the particles.

The generation of aerosols from liquids, solutions, or suspensions can be accomplished with various techniques. Aspirators, which use a pressure drop across an orifice and a stream of pressurized air to create droplets, have been widely used for generating liquid aerosols. These are usually combined with an impaction surface to limit the particle sizes produced. A typical example of such an air pressure-driven nebulizer is the Vaponephrin nebulizer, which uses a glass sphere directly opposite from an airstream jet passing over a capillary tube to create monodisperse aerosols. Ultrasonic vibrations from a piezoelectric crystal have also been used to fractionate liquids in a nebulizer apparatus. The most common liquid aerosol-generating technique is the spinning disk or top. These use centrifugal force to separate particles from a rotating flat surface upon which the test liquid is applied. Such apparatus are capable of producing highly monodisperse aerosols. Another technique for creating monodisperse aerosols is based on the controlled condensation of heated vapors on suitable nuclei. Most of the above methods can also produce dry particles by adding dry or heated air to the droplets after they are generated.

2. TERATOGENICITY

Robert H. Ross

2.1 INTRODUCTION

The evaluation of agents for teratogenic effects and their impact on human health is an area that only recently has become a significant regulatory concern. As Wilson (1979) states, "The notion that chemical and physical agents in the environment need to be tested for their potential to cause teratogenic effects when there is likelihood of exposure during pregnancy is a relatively new concept." It was not until the thalidomide tragedy in the early 1960s that the world's attention was focused on the fact that the human embryo is not isolated in an impervious maternal body where it is shielded from all but genetic harm. Wilson points out that the year 1966 probably deserves special recognition because of the significant efforts initiated at that time to minimize the risks to the unborn population from radiation (by the International Committee for Radiation Protection Recommendations) and to minimize such risks from drugs (by the Food and Drug Administration Guidelines).

This chapter will be primarily limited to a discussion of chemically induced congenital malformations and functional impairment. The first section will discuss some basic test parameters, the second section will examine the influence of the time of administration with regard to inducing teratogenicity, the third will explore the science of behavioral teratogenicity, the fourth will discuss the choice of species for teratological testing, and the last section will address some of the recent approaches in teratogenicity testing. The terms embryolethality or fetolethality will refer to the death of the embryo or fetus, teratogenicity will refer only to malformed offspring (both morphological and behavioral), and embryotoxicity or fetotoxicity will refer to teratogenic, growth retardation, abortifacient, or intrauterine death responses of offspring to a chemical.

2.2 GENERAL EXPERIMENTAL CONSIDERATIONS

Some basic parameters of testing chemicals for teratogenicity include dosage (number, levels, and duration), positive controls, number of species, number of test animals per dose group, administration route, and fetal examination.

In reviewing teratogenicity studies with these parameters in mind, it is important to distinguish between screening studies and those aimed at the further delineation of a teratogenic effect or those designed to investigate the toxicology of a clinically used dose. The protocols used in the latter two instances will often be different from those for the screening study.

Most of the comments in the following subsections will be directed to the screening study. Table 2.1 presents a brief insight into the protocols used by different researchers when conducting screening studies. The selection of studies in this table was determined by searching the recent issues of (1) *Teratology*, (2) *Teratogenesis, Carcinogenesis, and Mutagenesis*, and (3) *Toxicology and Applied Pharmacology*.

2.2.1 Dosage—Number and Level

The Environmental Protection Agency (EPA) (USEPA 1982a, 1982b), the Organization for Economic Cooperation and Development (OECD) (OECD 1982), and the Food and Drug Administration (FDA) (USFDA 1982) each recommends the use of at least three doses. An examination of the data in Table 2.1 (from 20 relatively recent research efforts) indicates that most researchers are apparently testing at least three concentration/dose levels.

Ideally, the high dose level should produce either maternal toxicity (but not lethality), embryotoxicity, or fetotoxicity [Wilson 1975b; World Health Organization (WHO) 1967]. The low dose should permit normal embryonic development, and the intermediate dose(s) should produce malformed offspring if the test chemical is teratogenic (Collins and Collins 1976). Extrapolation between the low dose and the intermediate dose or doses would provide an indication of the effect/no effect range of the test chemical.

2.2.2 Dosage—Duration

Schardein (1976) states that, in general, acute dosing of a chemical results in a greater teratogenic insult than does prolonged dosing. One example is that of the anticancer and antibiotic drug, actinomycin D, in rats. Malformations were induced in 27.9% of the surviving offspring with a single dose of 200 μg/kg given on day 9 of gestation, but when the drug was given as ten daily injections of 25 μg/kg on each of days 0 to 9 of gestation, only 8.7% of the surviving offspring was malformed (Wilson 1966). Comparable results were seen with the number of dead and resorbed fetuses—32.5% were either dead or resorbed with the single 200-μg/kg dose but only 9.7% were dead or resorbed when 25 μg/kg was given on each of days 0 to 9 of gestation. Further experimentation demonstrated that the dam was sensitized by prolonged treatment with actinomycin D at high doses so that for the dam or her offspring even moderately teratogenic doses become lethal. The stimulation of drug-metabolizing enzymes in the liver microsomes by prolonged dosing was suggested as the possible mechanism of this phenomenon, which has been observed by Koppanyi and Avery in experiments with more than 100 drugs (Koppanyi and Avery 1966, as reported by Schardein 1976).

Similar results were demonstrated with the administration of the benzhydrylpiperazine antihistamine drug chlorcyclizine (King et al. 1965).

Table 2.1. Literature Survey of Experimental Parameters in Testing of Chemicals for Teratogenicity

Chemical	Species—number per group	Number of doses/concentrations, duration, and route[a]	Extent of fetal examination	Positive control	Reference
Concanavalin A	Rabbit—7 to 11	1 dose level—160 μg intracoelomically	External (all live fetuses), skeletal (all live fetuses), soft tissue (internal organs of all live fetuses examined grossly and selected fetuses prepared for visualization)	No	DeSesso 1979
Indole-3-acetic acid	Rat—26 to 40; mouse—25 to 38	4 dose levels—5, 50, 200, or 500 mg/kg by gavage on gestation days 7	External (all fetuses), skeletal (all fetuses), soft tissue (1/3 of fetuses)	No	John et al. 1979
Octoxynol-9	Rat—25	2 dose levels—0.5 or 5 mg/kg/day intravaginally on days 6 to 15 of gestation	External, (all fetuses), skeletal (2/3 of litter), soft tissue (1/3 of litter)	No	Saad et al. 1984
Chlordiazepoxide (Cdz) and amitriptyline (Amt)	Hamster—11 to 18	1 dose level—28.5 mg/kg Cdz HCL or 70.3 mg/kg Amt HCL or combination intraperitoneally on day 8 of gestation	External, skeletal, and soft tissue (all fetuses)	No	Beyer et al. 1984
Tridemorph	Rats—21 to 27; mice—22 to 27	3 dose levels—20.6, 60.2, or 189.2 mg/kg for rats and 27.5, 81.7, or 245.1 mg/kg for mice on days 6 to 15 of gestation	External (all fetuses), skeletal and soft tissue (proportions not specifically stated)	No	Merkle et al. 1984
Tetrachlorobenzene isomers	Rat—10	3 dose levels—50, 100, or 200 mg/kg by gavage on days 6 to 15 of gestation	External (all fetuses) skeletal (2/3 of litter), soft tissue (1/3 of litter)	No	Kacew et al. 1984
ONO-802[b]	Rabbit—19 to 23	3 dose levels—0.0125, 0.0625, or 0.25 mg/kg intravaginally on gestation days 6 to 18	Skeletal (1/2 of fetuses), soft tissue (1/2 of fetuses)	No	Petrere et al. 1984

Table 2.1 (continued)

Chemical	Species—number per group	Number of doses/concentrations, duration, and route[a]	Extent of fetal examination	Positive control	Reference
Cyproheptadine chlorhydrate	Rat—7 to 61[c]	2 dose levels in one series (25 or 50 mg/kg) and 3 in another (15, 25, or 35 mg/kg); all given by oral intubation on gestation days 6 to 15	External (all fetuses); fetuses with abnormal appearance studied for soft tissue and skeletal defects, other fetuses randomly selected for soft tissue or skeletal examination	No	Rodriguez-Gonzalez et al. 1983
Hexamethylmelamine	Rat—15 to 30; Rabbit—15	Rat: 3 dose levels—10, 20, or 40 mg/kg by gavage on gestation days 6 to 15 or for 4-day periods during organogenesis; rabbit: 3 dose levels—20, 40, or 60 mg/kg by gavage on gestation days 6 to 18	Rat—external (all fetuses), skeletal (~2/3 of fetuses), soft tissue (~1/3 of fetuses); rabbit—all fetuses examined for external skeletal and soft tissue malformations	No	Thompson et al. 1984
Trimethyl phosphite	Rat—25	3 dose levels—16, 49, or 164 mg/kg by gavage on gestation days 6 to 15	External (all fetuses), skeletal (2/3 of fetuses), soft tissue (1/3 of fetuses)	Yes	Mehlman et al. 1984
Triamcinolone	Rat—3	3 dose levels—0.125, 0.25, or 0.5 mg/kg intramuscularly on gestation days 9 to 11, 12 to 14, or 15 to 17	External (all fetuses), skeletal (1/2 of fetuses), soft tissue (1/2 of fetuses)	No	Rowland and Hendrickx 1983
Methyl chloride	Rat—25; mouse—33	Rat: 3 concentrations - 100, 500, or 1500 ppm by inhalation on gestation days 7 to 19 (6 h/day); mouse: 3 concentrations—100, 500, or 1500 ppm by inhalation on gestation days 6 to 17 (6 h/day)	Skeletal (1/2 of fetuses), soft tissue (1/2 for head and fetal trunk examination)	No	Wolkowski-Tyl et al. 1983

Table 2.1. (continued)

Chemical	Species - number per group	Number of doses concentrations, duration, and route[a]	Extent of fetal examination	Positive control	Reference
Acetonitrile	Hamster—6 to 12	4 concentrations/dose levels—1800, 3800, 5000, or 8000 ppm by inhalation on gestation day 8; 100, 200, 300, at 400 mg/kg by intubation or intraperitoneally on gestation day 8	External (all living fetuses), ratio of fetuses examined for skeletal and soft tissue defects not given	No	Willhite 1983
Delalutin	Mouse—11 to 14[d]	3 dose levels—42, 416, or 833 mg/kg sc on gestation days 6 to 15	External (all fetuses), skeletal (~1/2 of fetuses), soft tissue (~1/2 of fetuses)	Yes	Seegmiller et al. 1983
Cocaine hydrochloride	Rat—8 to 12[d]; mouse—8[d]	Rat: 3 dose levels—50, 60, or 75 mg/kg mg/kg intraperitoneally on gestation days 8 to 12; Mouse: 1 dose level—60 mg/kg intraperitoneally on gestation days 7 to 16	External (all fetuses), 3:1 ratio for fetal examination of soft tissue to skeletal	No	Fantel and Macphail 1982
Valproic acid	Mouse—5 to 21	5 dose levels—1.0, 1.2, 1.4, 1.65, or 2.7 mmole/kg intraperitoneally on days 8 to 10 of gestation; 2 dose levels - 1.0 or 4.4 mmole/kg on days 11 to 13 of gestation	All live fetuses examined for external, skeletal, and soft tissue abnormalities	No	Kao et al. 1981
2-Nitro-p-phenylene diamine	Mouse—25 to 39	7 dose levels—32, 64, 128, 160, 192, 224, or 256 mg/kg sc on gestation days 6 to 15	External (all live fetuses), skeletal (all live fetuses), soft tissue (at least 1/3 of each litter, as well as all stunted fetuses and those with external malformations)	No	Marks et al. 1981

Table 2.1. (continued)

Chemical	Species - number per group	Number of doses concentrations, duration, and route[a]	Extent of fetal examination	Positive control	Reference
Phencyclidine	Mouse—18 to 45	4 dose levels—60, 80, 100, or 120 mg/kg by gavage on gestation days 6 to 15	External (all live fetuses), skeletal (all live fetuses), soft tissue (at least 1/3 of each litter, as well as all stunted fetuses and those with external malformations)	No	Marks et al. 1980
o-Toluenediamine	Rat—22 to 25; rabbit—14 to 16	Rat: 4 dose levels—10, 30, 100, or 300 mg/kg orally on gestation days 6 to 15; rabbit: 4 dose levels—3, 10, 30, or 100 mg/kg orally on gestation days 6 to 18	Skeletal (1/2 of each litter), soft tissue (1/2 of each litter)	Yes	Becci et al. 1983
Acrylonitrile	Hamster—NG[e]	4 dose levels—0.09, 0.19, 0.47, or 1.23 mmole/kg intraperitoneally on day 8 of gestation	Skeletal (all live fetuses)	No	Willhite et al. 1981
L-Azetidine-2-carboxylic acid	Hamster—5 to 16[d]	4 dose levels—100 mg/kg on gestation days 7 to 12; 200 mg/kg on gestation days 7 to 9, 7 to 12, or 10 to 12; 300 mg/kg on gestation days 9, 10, or 11; 600 mg/kg on gestation days 7, 8, 9, 10, 11, or 12; all doses administered intraperitoneally	External (all live fetuses), skeletal (1/3 of each litter), soft tissue (10 fetuses from remaining specimens in each group)	No	Joneja 1981

[a] Although negative controls not specified, each experiment used them.
[b] Synthetic E, postaglandin.
[c] All but the high dose group had at least 20 animals.
[d] Number of litters examined.
[e] NG = not given.

Chlorcyclizine hydrochloride administered to pregnant rats in 25-mg/kg doses over a 4-day period (days 12 to 15 of gestation) produced 16% malformed young; the same dosage given over a 15-day period (days 1 to 15) resulted in malformations in only 2% of the offspring. The results were even more striking when a 50-mg/kg dose was administered. Treatment on days 10 to 15 of gestation resulted in 82% malformed individuals, but only 0.1% malformed offspring was produced when a dosage of 50 mg/kg was given over days 1 to 15 (implantation was not inhibited). A subsequent paper (King et al. 1972) showed that the explanation behind this lack of teratogenic activity from prolonged treatment (defined in this instance as a 15-day period) of chlorcyclizine was the drug's self-stimulation of its own metabolism.

Wilson (1975b) and the WHO (1967) also report that the repetitive administration of certain chemicals during pregnancy can alter or mask a teratogenic action because of the ability of these chemicals to change their own metabolism. Wilson states three ways that some chemicals can change the rate of their own metabolism: (1) the induction of catabolizing enzymes (microsomes) in the liver or other tissues, which increases metabolism (also mentioned above); (2) the inhibition of naturally present enzymes which degrade chemicals; and (3) the induction of impaired function or overt pathology in important homeostatic organs such as the liver or kidneys. He further states that it is assumed these changes can occur within three or four days of the beginning of repeated treatments.

What then is the optimum dosing period for testing chemicals for teratogenicity? The foregoing paragraphs suggest that dosing for only one day or for short periods of three to four days during organogenesis would enable detection of chemical teratogens whose teratogenicity might go undetected if dosing duration was increased. Wilson (1975b) recommends both short-term and repeated tests. His recommended procedure consists of dividing the period of organogenesis into shorter dosage periods of three to four days but also dosing some animals throughout organogenesis. He states that fewer animals would be needed in each dose group than if dosage were given throughout organogenesis because the range of likely developmental defects, and hence overall variability, would be reduced by the shorter treatment span. As mentioned, some animals would still be dosed throughout organogenesis to detect any teratogenic effects that would result from the cumulative action of the chemical. Collins and Collins (1976) list three reasons why the single-dose technique—which, they report, is recognized as the most successful in producing abnormalities—is not used in place of the repetitive dosing method in screening studies. These are: (1) the necessity of treating different animals for each day of organogenesis increases the cost of the experiment, (2) without prior knowledge of the compound's effects, there is no way of predicting whether a specific organ or the entire developmental sequence will be affected, and (3) compounds that are likely to be tested for teratogenic action are usually encountered by humans as repetitive doses, and with single dosing cumulative effects cannot be measured.

Current guidelines (OECD 1981, USEPA 1982a, 1982b, USFDA 1982) recommend dosing during the period of organogenesis. The critical exposure periods for induction of behavioral changes in the postnatal animal are at present not well defined, and it is conceivable that existing guidelines will be amended as more information becomes available.

2.2.3 Positive Controls

Although positive controls are not recommended in current guidelines for teratogenicity testing (OECD 1981, USEPA 1982a, 1982b, USFDA 1982), they have been used to demonstrate that the test animal will produce malformed offspring after exposure to an established teratogenic chemical as well as to validate the soundness of a specific laboratory's or researcher's methodology. This use of positive controls in teratology has not been completely abandoned (Table 2.1).

The data generated from positive controls must be evaluated with some caution because an animal species can be very sensitive to one teratogen but only mildly or not at all sensitive to another. Table 2.2 shows the teratogenic effects from thalidomide in several animals, including man. Man, monkeys, and rabbits are shown to be susceptible to the teratogenic action of thalidomide, but several strains of rats, mice, and hamsters are not (at least by conventional testing protocols). The action of the human teratogen aminopterin (Table 2.3) also shows apparent species variability. Teratogenic effects have been seen in man and mice but not in rats and monkeys (Table 2.3). The data in these tables clearly demonstrate that an animal may be susceptible to the teratogenic action of one chemical but not necessarily to that of another chemical. Therefore, if a positive control is established in the animal species being tested and the test chemical does not produce teratogenic effects, it cannot be assumed that the test chemical would not induce teratogenic effects in another animal species or even another strain of the same species. Conversely, if an established teratogen such as thalidomide fails to induce teratogenicity in an animal being used as a positive control, it cannot be assumed that the test chemical will not produce teratogenic effects in the same species.

2.2.4 Number of Species

As Tables 2.2, 2.3, and 2.4 indicate, the choice of species or strain can determine whether or not a chemical is identified as a teratogen. Species variability to potential teratogenic agents would thus dictate that at least two species should be used. Current EPA guidelines recommend the use of at least two species, with the rat and rabbit being the preferred species when only two species are used (USEPA 1982a, 1982b). The FDA guidelines (USFDA 1982) also state that these are the preferred species.

Table 2.2. Thalidomide Action in Various Species

Animal	Species strain	Dose, route and duration	Effects	Reference
Man		Dose not specified, oral administration during first trimester	Missing limbs; auricle or pinna of ear decreased in size; lesser deficiencies	Smithells 1962
		Dose and duration not known; oral administration	Reports of several cases—hypoplasia and aplasia of the extremity or individual bones, absence and malformation of limbs; malformations of other organs such as stenoses and malrotation of the gastrointestinal tract and dysplasias of the external ears and eyes often associated	Pfeiffer and Kosenow 1962, Lenz and Knapp 1962
Mouse	Schofield	400 mg/kg orally throughout pregnancy	No malformed fetuses in albino mice	Somers 1963
	A, C$_3$H, Swiss	A-31 to 625 mg/kg/day[a]; C$_3$H, Swiss - 62 mg/kg/day; administered orally on days 6 to 7 of gestation and continued for 4 to 6 days	Skeletal malformation; open eye; enlarged skull; curvature of back; kinky tail; phocomelia; micromelia	DiPaolo et al. 1964
	CF$_1$, ICR, C$_{57}$, CBA, SJL	200 mg/kg total given orally for gestation days 2 to 12	No malformed offspring but increased resorptions in CF$_1$ strain	Fratta et al. 1965
Rat		2 g/kg total ip[b] on gestation day 12, days 11 and 12, or days 12 and 13	16 litters - two had abnormalities, complete resorption in one, more than 50% resorption in three, and normal offspring in ten; abnormalities consisted of stunting of extremities and tails, absence of dorsal and lumbar vertebrae and ribs, absence of digital bones, and curved bones of the fore and rear extremities	Murphy 1962

Table 2.2 (continued)

Animal	Species strain	Dose, duration, and route	Effects	Reference
	Wistar	200 or 400 mg/kg total given orally throughout pregnancy	Fetal resorptions but no abnormalities	Somers 1963
	Wistar	45 mg/kg iv^c on day 10, 11, or 12 of gestation	Skeletal deformities of thoracic ribs and of spinal column in 33 and 56% of fetuses on day 11 and 12, respectively; deformities of eyeball as a result of administration on day 10 or 12	Parkhie and Webb 1983
	Sprague-Dawley	10 (ip), 20 (orally), 50 mg (orally or ip) per day at various dosing schedules between days 7 and 17 of gestation	520 young examined—36 grossly malformed (6.9%), 40 showed malformations after clearing of fetuses, 76 total malformations (14.6%); malformations included malrotation of hind limbs, hamartoma of the palate with accessory incisors, lack of a tail in one instance, and a subcutaneous cartilaginous-type mass of tissue from the middorsal region to the tail	King and Kendrick 1962
	Charles River	100 mg/kg 6 to 12 total given orally on gestation days 6 to 12	Resorptions but no malformations	Delahunt et al. 1966
	Long Evans and Dunning Fischer	150 mg/kg total given orally on gestation days 3 to 12	No apparent injury	Fratta et al. 1965
	Holtzmann	25-500 mg/day orally or ip at various dosing schedules during organogenesis	More resorptions than controls; only one fetus showed serious limb malformation: significant teratogenic effect shown by missing sternebrae and delayed ossification of the sternum; malformations different from humans	Moore 1965

Table 2.2 (continued)

Animal	Species strain	Dose, duration, and route	Effects	Reference
Hamster	Not given	Up to 8000 mg/kg throughout pregnancy	Not teratogenic	Somers 1963
	Not given	150 mg total given orally for gestation days 3 to 12	Not teratogenic	Fratta et al. 1965
	Inbred and random	350 mg/kg/day orally throughout pregnancy	Inbred lines showed 6.2% incidence of grossly malformed fetuses—acrania or split cranium, abnormal positioning of legs, kinking of tail, and cleft palate; random bred lines showed no significant teratogenicity	Homburger et al. 1965
Monkey	Rhesus and stump-tailed macaque	5 mg/kg/day orally on gestation days 26 to 28 or 24 to 30 or 10 mg/kg on days 25 to 27, 24 to 30, or 23 to 29	Severe deformities in most species—missing digits, missing radii and ulnae, shortened humeri, kinking and in some cases shortening of tail	Vondruska et al. 1971
	Bonnet	10 mg/kg orally on day 24, 25, 26, 27, 28, or 29 of gestation; 30 mg/kg orally on day 25 or 28	Temporal and mandibular bones malformed	Hendrickx and Newman 1972
	Baboon, bonnet, and cynomolgus	17.5-300 mg/kg total given orally at various dosing schedules on gestation days 18 to 43 (both single and multiple doses)	Malformed fetuses - deformed limbs, spina bifida, kinked tail, etc	Hendrickx 1970
	Marmoset	25 mg/kg/day orally on gestation days 38 to 52	Reduction in size of spinal cord and dosal root ganglia; pattern of deformities said to be similar to man	McBride and Vardy 1983

Table 2.2 (continued)

Animal	Species strain	Dose, duration, and route	Effects	Reference
Rabbit	New Zealand white and Dutch-Belted	Not given	Low incidence of deformed appendages in New Zealand whites; increased incidence of limb defects in Dutch-Belted—agenesis or hypogenesis of the ulna, radius, tibia, and/or fibula; absence of some bones in limbs	Delahunt 1965
	New Zealand white	150 mg/kg/day orally on days 8 to 16 of pregnancy	Limb deformities in almost every litter	Somers 1963
	New Zealand white	150 mg/kg/day orally on gestation days 8 to 16 or 2 to 9	Sixty-seven percent of kits deformed when administered on days 8 to 16—most predominant effects seen in limbs (arthrogryposis, micromelia, absence of digits), but developmental failure of visceral organs (kidneys, adrenals) and in bony structures also observed; only 17% abnormalities in the one litter exposed on days 2 to 9	Drobeck et al. 1965
	Japanese white	50, 150, or 300 mg/kg/day orally on gestation days 6 to 18	External skeletal and internal anomalies significantly ($P<0.01$) different from controls at 150 and 300 mg/kg day; external anomalies included contracture of forearm and club foot	Matsubara et al. 1983

Table 2.2 (continued)

Animal	Species strain	Dose, duration, and route	Effects	Reference
	Japanese white—NIBS	50, 150, or 300 mg/kg/day orally on gestation days 6 to 18	External, skeletal, and internal anomalies significantly different at all dose levels ($P<0.01$); external anomalies of highest frequency were holoprosencephaly, anencephaly, hypoplasia of ala nasi, and club foot	Matsubara et al. 1983

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[a] 625 mg/kg dose resulted in 55% abortion rate and thus a minimum number of malformations resulted.

[b] Intraperitoneally

[c] Intravenously

Table 2.3. Aminopterin Action in Various Species

Animal	Species/ strain	Dose, route, and duration	Effects	Reference
Man		29 mg orally over 10 days at ~6.5 to 8 weeks of gestation	Excessively large head; nasal bridge broad and flattened; eyes widely separated; malformed ears; mandibular hyoplasia; posterior cleft palate; absence of parietal bones in skull	Emerson 1962
		20 mg orally over several weeks during second trimester of pregnancy	Multiple skull anomalies; left talipes equinovarus	Meltzer 1956
		12 mg orally over 12 days during early pregnancy	Numerous head abnormalities—soft skull, no ossification of parietal bones, eyes wide apart, broad nasal bridge, posterior cleft palate, and others; large hands, other anomalies also described	Warkany et al. 1959
Mouse	ICR-DUB	25 mg ip[a] on day 11 or 12 of gestation	Short or small limbs; hemimelia; severe ectrodactly (absence of one or more fingers or toes)	Kochhar 1975
Rat	Wistar and others	0.05, 0.075, 0.1, or 0.2 mg/kg single ip injections on various gestation days	Injections on days 7 and 10 effective—embryolethality	Baranov 1965
	Wistar	0.1 mg/kg ip on gestation day 11 or 12	No malformed offspring—fetuses resorbed with high doses	Murphy 1962
Monkey	Rhesus	0.12 mg/kg orally on days 24, 25, and 26 of gestation	No injury to offspring	Tanimura 1972
	Rhesus and cynomolgus	0.1-0.2 kg/day on gestation days 21 to 33; 1.0 kg/day on gestation days 38 to 39	Two aborted and one normal fetus, but no malformed offspring	Wilson 1969
Rabbit	Not given	2 mg/kg by injection to six-day-old blastocyst	No injury	Hay 1964

[a] Intraperitoneally.

Table 2.4. Methotrexate Action in Various Species

Animal	Species/ strain	Dose, route, and duration	Effects	Reference
Man		5 mg/day orally through second month of pregnancy	Major abnormalities of skull of infant	Powell and Ekert 1971
		2.5 mg/day orally for five days ~ between 8th and 10th week of pregnancy	Major anomalies—absence of frontal bone, absent lambdoid and coronal sutures, multiple anomalous ribs, unusual facies, and absence of all digits on right foot and all but one on left foot	Milunsky et al. 1968
Mouse	ICR	0.3 to 50.0 mg/kg ip[a] on day 10 of gestation	25 and 50 mg/kg produced congenital defects, primarily cleft palate and reduction of digits	Skalko and Gold 1974
Rat		0.2 mg/kg on day 9 of gestation	103 embryos exposed—64% resorptions and 30% malformations	Wilson 1971b
Monkey	Rhesus	2.5 to 4 mg/kg various regimes on gestation days 17 to 45	Most fetuses normal, three aborted and one showed moderate gut rotation; only 13 embryos exposed, so trivial teratogenicity might reflect small sample size	Wilson 1971b
	Rhesus	3 mg/kg iv[b] on gestation days 29 to 32	Mildly embryolethal, nonteratogenic, transitory growth retardation	Wilson et al. 1979
Rabbit	New Zealand white	9.6 mg/kg iv on day 10 of gestation	50% fetal mortality and a 25% malformation rate in surviving fetuses	Jordan et al. 1970
	New Zealand white	19.2 mg/kg iv during days 11 to 14 of gestation	Multiple anomalies—facial clefts, cleft palates, defects of fore and hind limbs, etc.	Jordan 1973

[a] Intraperitoneally.

[b] Intravenously.

An economical alternative would be to test the two species separately instead of concurrently and test the second species only when the first species has given negative or ambiguous results. In the interest of human safety, the test chemical would have to be considered potentially teratogenic to humans if one animal species produces malformed offspring. However, the more animal species that demonstrate a chemical to be teratogenic, the greater the possibility that the chemical will be teratogenic in humans, and by testing two species data would be available for species comparisons. Such information as the difference or similarity in dose or anatomical defects would become available and would be beneficial in other teratogenic studies with these same species. In addition, the use of two species would give a better understanding of the effect/no effect level of the test chemical.

The use of only two species (a rodent and rabbit) appears to be justified in view of the fact, stated by the WHO in 1967, that all substances shown to be teratogenic in man have also demonstrated teratogenic activity in the mouse, rat, and rabbit. The WHO cautions, however, that negative results obtained by testing chemicals in these species provide no absolute assurance that the chemical will not induce teratogenic effects in man. This situation would particularly be of concern when the chemical tested is a drug intended for use during human pregnancy or a chemical to which heavy exposure is likely for pregnant women. In these instances, when the rabbit or rodent does not demonstrate teratogenicity, a third species closer to man physiologically probably should also be used as a test animal. This is similar to the testing scheme proposed by Wilson (1975), who recommends the use of the rat, mouse, hamster, or rabbit as screening species to establish the embryotoxic dose range and not as an end point for teratogenic studies unless appreciable embryotoxicity is demonstrated at appropriate multiples of the anticipated human dose.

2.2.5 Number of Test Animals Per Dose Group

The number of pregnant animals per dose group is an important consideration. As Weil (1970) points out, for a statistical analysis of the results of an experiment designed to assess teratogenicity, the number of independent sampling units, N, is the number of dams or litters. Using the number of pups as N will produce invalid conclusions.

The number of animals used to test chemicals for teratogenicity is variable. The results of some of the studies cited in Table 2.1 might be questioned because too few animals in some dose groups were used to permit reliable statistical analysis. The WHO (1967), although not specifying animal numbers, states that the number of rodents used must be large enough to satisfy statistical requirements. For species more closely related to man, the WHO recommends that the number of animals be as large as practicable, in order to obtain reproducible results. Current guidelines recommend 20 pregnant rats, 20 pregnant mice, 20 pregnant hamsters, and 12 pregnant rabbits per dose group (OECD 1981, USEPA 1982a, 1982b, USFDA 1982). The FDA guidelines add that these are minimum numbers of animals at or near term.

2.2.6 Administration Route

The choice of administration route is usually dictated by the present or expected human exposure to the test chemical (Collins and Collins 1976). In most instances, the chemical should be administered orally, but administration by other methods such as dermal or inhalation may be necessary in order to simulate human exposure. The EPA (USEPA 1981a, 1982b), the FDA (USFDA 1982), and the OECD (OECD 1981) guidelines recommend oral administration by gavage unless the chemical and physical characteristics of the test chemical or the use pattern would suggest another route.

2.2.7 Fetal Examination

Current guidelines (OECD 1981, USEPA 1982a, 1982b, USFDA 1982) are in agreement concerning the extent of fetal examination. All fetuses should be examined externally, and one-third to one-half of all rat, mouse, and hamster fetuses should be examined for skeletal defects with the remainder examined for soft tissue malformations. All rabbit fetuses should be examined for both skeletal and soft tissue malformations. The data in Table 2.1 indicate that, while many researchers are following this protocol, some are not (e.g., Mehlman et al. 1984).

2.3 STRUCTURE-ACTIVITY RELATIONSHIPS

As Schardein (1983) states "One of the most potentially promising measures of teratologic risk is the structure-activity relation of chemicals." Schuler et al. (1984) in subjecting 15 glycol ethers to an in vivo reproductive toxicity assay found that the 5 ethers with terminal methyl groups and 2 with terminal ethyl groups produced few viable CD-1 mice litters whereas 3 butyl ethers, 3 glycol ethers with terminal hydroxy groups, and 2 ethyl ethers did not produce such profound fetotoxicity.

The evidence generated by Willhite et al. (1984) in their study of the structure-activity relationships of retinoids suggested that the changes in teratogenic activity associated with structural modification of vitamin A at carbon 15 were primarily dependent upon the presence of or biotransformation to a free carboxyl or a moiety with an equivalent pKa at carbon 15. The molecular size of the substitutent or the sterochemical position about carbon 13 was considered of secondary importance for the induction of terata.

A series of papers (Keeler 1970, Brown 1978, Brown and Keeler 1978) have discussed the teratogenic structural requirements of naturally occurring steroids. Keeler (1970) studied jervine, 11-deoxojervine, and 3-glucosyl-11-deoxojervin, three compounds teratogenic to sheep (causing clyclopia) which structurally differ only in substitution at the 3 and 11 positions. Keeler tentatively concluded that the nature of the substitution of these two positions has little influence on teratogenicity. Testing with more than 18 additional compounds including rubijervine, veratramine, and testosterone in sheep did not produce cyclopia, although veratramine did cause occasional bowing of limbs, joint flexure, and lack of muscular control. None of these compounds possessed the fused oxide and piperidine rings present in the active compounds (i.e., jervine, 11-deoxojervine, and 3-glucosyl-11-deoxojervine).

Brown (1978) investigated the teratogenic activity in the hamster of several derivatives of jervine and 11-deoxojervine. His findings showed that highly teratogenic compounds present a negatively charged center accessible to the steroid α face. Similarly, Brown and Keeler (1978) stated that "conventional steroids with a secondary basic nitrogen bonded in a position analogous to hormone binding sites (accessible to the α face of the steroid) would be predicted to be substantially teratogenic."

Freese et al. (1979) studied the correlation between the growth inhibitory effects, partition coefficients, and teratogenic effects of a number of lipophilic acids and found many of the acids that were potent inhibitors of mammalian cell replication to be also teratogenic.

Schumacher (1975) reported that very strict structural requirements govern the teratogenic effects of thalidomide (in particular an intact phthalimide or phthalimidine moiety) and, although investigations with thalidomide have revealed a number of interesting and rather unique properties of this chemical, no conclusive evidence connecting any special property of thalidomide with its teratogenicity has been presented.

2.4 TERATOGENESIS AND TIME OF ADMINISTRATION

A chemical should be considered an unlikely teratogen only after proper teratological experimental procedures have been performed. One of the most critical aspects of testing chemicals for teratogenicity is determining the most susceptible time during gestation for chemical administration.

2.4.1 Preimplantation

In both rat and man, there is an interval of approximately six days between the fertilization of the oocyte and the implantation in the endometrium known as the preimplantation or predifferentiation stage (Leonard 1983; also see Table 2.5). In this largely undifferentiated state, the ovum is generally considered to not be at risk from teratogenic agents. With respect to drugs Leonard (1983) states, "It appears that drugs are either toxic to the entire embryo, thereby resulting in its death, or affect only a relatively small number of cells, that can be repaired without causing any obvious physical damage." However, consideration must be given to the possibility of chemicals with long half-lives whose effects may be manifested days after exposure. An example is the progestin cyproterone

Table 2.5 Preimplantation Developmental Stages in Man and Laboratory Animals
Figures as Postconceptional Days

Stage	Human	Monkey & baboon	Rabbit	Rat	Mouse
Fertilzed eggs in the fallopian tubes	1	1	1/4	1	1/2
2 cells in oviduct	2	2	1/2	2	1
4 cells in oviduct	2 1/2	3	1	3	2 1/4
8 to 12 cells in oviduct	3	4	2	3 1/4	2 1/2
Blastocele begins to develop - oviduct	4	5 to 6	3	4	3
Free blastocyst in uterus	5	7 to 8	4	5	4
Implanting blastocyst	6	9	7	6	4 1/2
Implantation in progress	6 to 13	9 to 15	7to 8	6 to 8	4 1/2 to 6
Post implantation[a]	19 to 260	18 to 160	8 to 29	9 to 21	7 1/2 to 19
Delivery	260 to 280	164 to 168	30 to 32	22	20

[a] The following days are those of high teratogenic risk: human, 19 to 80; monkey and baboon, 18 to 50; rabbit, 8 to 18; rat, 9 to 17; mouse, 7 1/2 to 15.

Source: Adapted from Leone 1977, Fig. 1, p. 19.

acetate whose long biological half-life (60 h) was responsible for abnormal morphological development when administered in high doses on day 2 of gestation in the mouse (Eibs et al. 1982).

With respect to behavioral teratogens, limited evidence suggests that exposure during the predifferentiation period may result in behavioral abnormalities. Werboff and Havlena (1962) found that tranquilizers administered to the gravid rat induced behavioral changes in the offspring independent of the trimester of gestation during which these drugs were given. In this study, the tranquilizers reserpine, chlorpromazine, and meprobamate (0.1, 6.0, and 60.0 mg/kg/day subcutaneously, respectively) were administered on days 5 to 8, 1 to 14, or 17 to 20 of gestation with each daily dose divided into three equal injections. Because day 8 (Leonard 1982) or day 9 (see Table 2.5) in the rat is considered the first day of the differentation phase of development, administration on days 5 to 8 would be during the predifferentiation phase.

2.4.2 Organogenesis

The period of embryological differentiation (organogenesis) is considered to be the most sensitive developmental stage to the action of teratogenic chemicals (Wilson 1975, Schardein 1976). Schardein (1976) lists what he considers to be the critical periods for a number of animal species (see Table 2.6); the period of greatest susceptibility in man is similar to that of the baboon and the rhesus monkey. As indicated in Table 2.1, dosing periods used by researchers vary. Current regulatory guidelines (OECD 1982, USEPA 1982a, 1982b, USFDA 1982) recommend appropriate dosing periods for the mouse, rat, hamster, and rabbit. These guidelines are consistent with respect to the mouse and rat (gestation days 6 to 15). The USFDA guidelines are slightly different with respect to the hamster and rabbit; the OECD and EPA recommend dosing during gestation days 6 to 14 for the hamster and 6 to 18 for the rabbit, whereas the FDA recommends days 4 to 14 for the hamster and 7 to 18 for the rabbit.

Although Table 2.6 indicates several days categorized as in the critical period, the embryologic differentiation of the various organs proceeds at varying rates; thus, the time of teratogenic insult is important with regard to which tissues are affected. This is illustrated in the research of King et al. (1972), Kajii et al. (1973), Sadler and Kochhar (1975), Inouye and Murakami (1977), and Eibs et al. (1982). Inouye and Murakami studied the teratogenicity in mice of the hair dye constituent 2,5-diaminotoluene and found that subcutaneous or intraperitoneal injections of 50 mg/kg body weight caused a low incidence of exencephaly (cranial malformation) and prosoposchisis (fissure of the face, e.g., hairlip) and a high incidence of skeletal malformations in those animals treated on day 8 of gestation. No such malformed fetuses, however, were found in those animals treated on days 10 to 14 of pregnancy, and only a very low incidence of vertebral and rib anomalies was observed in the fetuses treated on gestation day 7 or 9.

In studying the teratogenic effects of a single oral injection (intubation) of chlorambucil (14.2 or 20 mg/kg) on the 10th, 11th, 12th, or 13th day, Sadler and Kochhar (1975) observed limb defects from treatments on the 11th or 12th days of gestation and tail defects from treatment on all days. Treatment on the 13th day resulted in digital defects but without the long-bone defects observed with day 11 and 12 treatments; defects were observed only at the highest dose level. Sadler and Kochhar also found that in vitro limb bud response to chlorambucil was similar whether the limb buds were taken from pregnant mice after chlorambucil treatment or the chlorambucil was applied to limb bud cultures after removal from non-treated pregnant mice.

Eibs et al. (1982) administered cyproterone acetate (30 mg/kg subcutaneously) to mice on one of days 1 to 12 of gestation. The most sensitive gestation days for abnormalities were days 5 and 6 for the urinary tract, days 8 and 9 for the respiratory tract, and days 10 and 11 for cleft palate.

The significance of the critical period is also shown by the human teratogen thalidomide. One study identified 113 pregnant women who took thalidomide between August 1959 and December 1961, but only 7 women took the drug during the critical period (reported here to be between 34 and 50 days after the last menstrual period) (Kajii et al. 1973). Of these

Table 2.6. Critical Periods of Organogenesis in Animals

Species	Mean duration of gestation (days)	Gestation Days[a]
Hamster, golden	16	4 to 14
Mouse	19	7 to 16
Rat	21	9 to 17
Rabbit	31	8 to 21
Ferret	43	8 to 28
Cat	63	5 to 58; 5 to 15 most favorable
Dog	63	1 to 48; 8 to 20 estimated
Guinea pig	68	11 to 20
Pig	114	12 to 34
Sheep	150	14 to 36
Monkey, rhesus	168	20 to 45; 22 to 30 most susceptible
Monkey, baboon	175	22 to 47
Armadillo	225	1 to 30
Human	278	20 to 55
Cow	284	8 to 25
Horse	336	?

[a] Period of embryological organogenesis or period of known susceptibility.

Source: Adapted from Schardein 1976, Table 2-5, p. 17. Data collected from several sources. Reprinted with permission of the publisher.

seven women, three delivered malformed babies, and four delivered babies without apparent malformations. Possible explanations for the four normal babies include inaccurate pregnancy diagnosis time, so that thalidomide possibly was not taken during the critical period, or actual resistance to thalidomide. Two additional women took the drug before the critical period, on day 19 and day 23, respectively; both women aborted (induced and spontaneous abortions, respectively). The remaining 104 women took thalidomide after the critical period with all offspring apparently normal, except for one reported case of anal stenosis.

One other example of the importance of the critical period is shown in rats administered β-aminopropionitrile, a chemical that induces cleft palate (King et al. 1972). In this instance, there were a significant number of offspring with cleft palate only when day 15 of the gestation period was included in the dosing schedule. All of the viable fetuses had cleft palate when 875 mg/kg was given on day 15, or over days 13 to 15 or 14 to 15, but when a total dose of 4320 mg/kg was administered over days 12 to 14, only 8% of the young were malformed.

2.4.3 Histogenesis and Fetal Period

The period of prenatal development that slightly overlaps organogenesis but extends primarily into the fetal period is known as histogenesis (Fig. 2.1). Teratogenic agents that come into contact with the developing fetus during this time of tissue formation and development can cause minor structural deviations, but the abnormalities that are more likely to occur during the fetal period are those involving growth or functional aspects of development (Wilson 1975b). An example is the administration of vitamin A during the early- and mid-fetal period (Hutchings et al. 1973, Hutchings and Gaston 1974). Pregnant rats administered a teratogenic dose of vitamin A during the early fetal period (days 14 and 15 of gestation) produced offspring that showed a behavioral deficit indicating nonreinforcement (characterized by a decreased ability to inhibit responding to an auditory signal). In addition to behavioral effects, a generalized retardation in growth was induced, as evidenced by delayed onset of fur growth and eye-opening and by reduced body weight as well as reduced brain size with obvious microcephaly in one animal. Pregnant rats treated with vitamin A during the mid-fetal period (days 17 and 18 of gestation) produced offspring with no retardation in growth or in brain size, but with a possible motor deficit affecting coordination that resulted in slower rates of response to auditory stimuli than control animals.

2.5 BEHAVIORAL TERATOGENESIS

As described by Hutchings (1983), behavioral teratology [Vorhees (1983) uses the synonym *psychoteratology*] is an integration of teratology with experimental psychology. The primary concern of behavioral teratology is the study of neurobehavioral changes that occur when germ cells,

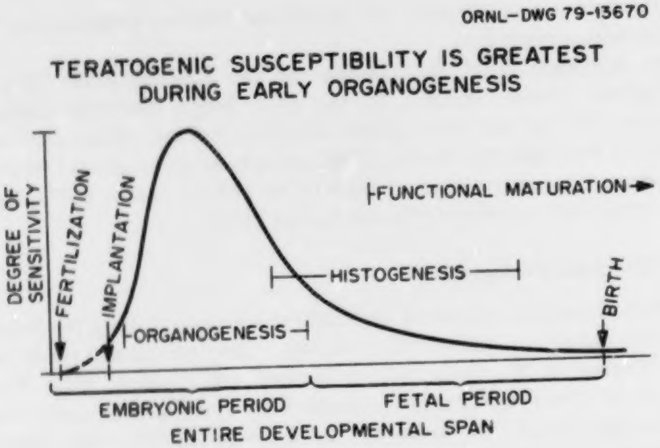

Figure 2.1. Representation of the susceptibility of the human embryo to teratogenesis, beginning with fertilization and continuing throughout intrauterine development. (Source: Adapted from Wilson 1975b, Figure 4, p. 662).

embryos, fetuses, and immature postnatal organisms are exposed to a variety of environmental disturbances and events. Regarding the state of the art of behavioral teratology, Hutchings (1983) writes, "I should emphasize, however, the current state of behavioral teratology as a fledgling discipline; its subject matter remains largely unexplored and uncharted, its methodology has been uneven and occasionally wanting, and its accomplishments strike a tenuous balance between false starts and palpable inroads made by a few pioneering researchers. But despite its immaturity and tentative beginnings, there should be no doubt that it is emerging as a new scientific speciality."

Although the period of organogenesis is generally considered the time the embryo is at greatest risk to morphological alteration, the exposure period is not as well defined with respect to the induction of behavioral changes. As discussed in Section 2.4.3, the histogenesis period can be a sensitive period for behavioral teratogens. However, as Coyle et al. (1976) reported, behavioral anomalies can also be induced in the offspring as a result of exposure during organogenesis. They cite Hoffeld and Webster (1965) and Murai (1966), who found that when chlorpromazine was administered during early pregnancy maze learning was impaired, but no effects were observable when it was applied late in pregnancy. In addition, Werboff and Havlena (1962) demonstrated that behavioral changes induced by the administration of tranquilizing drugs are independent of the trimester of gestation. Compounding the situation even further are studies by Gauron and Rowley (1969) and Friedler (1978) which show that the behavior of offspring can be affected even by pregestational exposure to certain chemicals.

An investigation of the holdings of the Environmental Teratology Information Center (ETIC) of the Oak Ridge National Laboratory indicates that of the over 25,000 citations indexed concerning various aspects of teratogenicity testing, 2645 are related to behavioral teratology. By species this breaks down as follows: rat (1078), mouse (297), hamster (11), rabbit (69), monkey (38), and human (1152).

2.5.1 Historical Perspective

Werboff and his coworkers are credited with establishing the branch of teratology known as behavioral teratology. They published several papers (e.g., Werboff et al. 1961a, 1961b; Werboff and Havlena 1962; Werboff and Kesner 1963) detailing the postnatal effects of prenatally administered psychotropic drugs. As indicated by Leonard (1983) and Hutchings (1983), it was not until the early 1970s that the work of Werboff and colleagues was extended. The realization that prenatal exposure can cause behavioral alterations has resulted in both Japan and Britain mandating that behavioral evaluations be conducted on all new drugs during premarket reproductive testing (Vorhees and Butcher 1982, as reported in Vorhees 1983). Although similar regulations have not been established in the United States, Vorhees (1983) cites several events which suggest that U.S. regulatory authorities are giving serious consideration to establishing guidelines for behavioral teratogenicity testing.

2.5.2 Behavioral Testing Methodologies

Because no single test gives a comprehensive behavioral assessment, a multitesting procedure is usually employed. Jensh (1983) in his review on behavioral testing procedures cites several authors (Spyker and Avery 1976, Kimmel 1977, Rodier 1978, Silverman 1978, Buelke-Sam and Kimmel 1979, Lasagna 1979, Vorhees et al. 1979) who have suggested criteria for an effective multitest procedure. These criteria are listed by Jensh (1983):

1. The tests should be simple.
2. The tests should be comprehensive, examining a variety of behavioral functions (global).
3. The tests should be sensitive to slight alterations in the organism's functions, as well as to alterations produced by a number of different agents.
4. The behavioral schedule should be simple, to minimize the training time of personnel.
5. The tests should take a minimum amount of time.

6. The tests should be reliable, reproducible, and valid in the same laboratory as well as in the laboratories of other investigators.
7. The tests should have a proven history of positive results.
8. The tests should be economical in terms of financial expense and utilization of animals.
9. The tests should be within the framework of existing guidelines.
10. The protocol should move in graded steps from elementary to more sophisticated tests.
11. Data should be quantifiable.
12. The tests should be predictive of effects in humans.

Table 2.7 lists what Jensh (1983) considers the more commonly used tests either singly or as part of a behavioral test battery. Earlier, Buelke-Sam and Kimmel (1979) reported on a survey they conducted concerning behavioral methods in teratologic evaluation used by those researchers who responded to their survey (Table 2.8). As both Tables 2.6 and 2.7 indicate, a variety of behavioral test methods are being used to assess behavioral alteration. This variety of test methods as well as the varying exposure regimens and postnatal environmental conductions used by researchers emphasizes the need for standardization of screening methods for behavioral teratology (Buelke-Sam and Kimmel 1979). Jensh (1983) states that "standardization of test procedures is of paramount importance" and adds that "the investigator needs to have access to a standardized procedure for each of the chosen tests, including experimental protocols, construction designs, techniques, past results, and interpretative implications and limitations." Standardization procedures are in fact under way (Kimmel et al. 1982, 1983; Kimmel 1984), and a workshop to present the data from a six-laboratory study was held on September 3-6, 1985, in Cincinnati, Ohio.

2.6 SPECIES COMPARISONS

Proper selection of a test species is important. The large number of animals necessary for toxicological research usually dictates that the test species be easily available and economical. The following subsections will consider some general aspects of species selection; will briefly discuss the advantages and disadvantages of the rat, mouse, hamster, rabbit, monkey, and other less commonly used species as models for extrapolation to man; and will compare the response of three known human teratogens—aminopterin, methotrexate, and thalidomide—with the response in test animals.

Table 2.7. Behavioral Testing Procedures[a]

I. Physical Development

- Pinna detachment
- Incisor eruption
- Eye opening
- Ear unfolding and opening
- Testes descent
- Vaginal opening
- Development of fur

II. Behavioral Analyses

A. Reflex development tests[b]

- Crossed extensor
- Flexor dominance
- Roofing reflex
- Limb withdrawal
- Forelimb placing
- Hindlimb placing
- Forelimb and hindlimb grasp
- Suckling ability
- Tail hang
- Vibrissae stroking
- Ear twitch
- Tail pinch
- Tremors
- Surface righting
- Air righting
- Auditory startle
- Negative geotaxis
- Visual placing
- Vibrissae placing
- Cliff avoidance
- Pain reflex reaction
- Olfactory reflex

B. Motor and coordination tests[c]

- Bar holding
- Forelimb hanging
- Visual orientation
- Inclined plane
- Rotarods
- Parallel bars
- Ascending wire mesh
- Ascending a vertical rod
- Clinging to and descending a vertical rope
- Swimming

C. Spontaneous or nonforced behavior tests[d]

- Spontaneous motor behavior
- Head elevation
- Head pointing
- Hindlimb elevation
- Rearing
- Pivoting
- Gait
- Crawl
- Walk
- Backward walking
- Grooming
- Circling behavior
- Sleeping and somnolence
- Hopping
- Homecage emergence
- Resistance to handling
- Feeding
- Undisturbed behavior
- Spontaneous and general activity
- Nose poke
- Head dip
- Step-down test
- Thirst test
- Maternal behavior
- Open-field
- Activity wheel

D. Forced behavior tests[e]

1. Mazes

- T-maze
- Y-maze
- Hebb-Williams maze
- Lashley III maze
- Olton maze

2. Operant conditioning tests

- Bar-(lever-) press
- Avoidance testing
- Discrimination reversal learning

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[a] Data compiled from Jensh 1983.

[b] Some of these include complex motor activity.

[c] Primarily include forced, complex activities.

[d] May include some motor evaluations.

[e] Primarily include learning skills.

Table 2.8. Behaviors Tested and Methods Used in Teratologic Evaluation[a]

Observation (23)[b]	
18[c]	general activity and condition
3	modified Irwin method
2	photography/video taping
1	residential unit
Reflex development (34)	
17	surface righting
15	auditor startle
11	pupillary contraction
10	air righting
6	cliff avoidance
6	touch-escape
6	visual placing
5	Fox method
4	nonspecified, animal
3	negative geotaxis
3	Smart & Dobbing method
2	nonspecified, human
1	stabilimeter
Motor coordination (35)	
18	rotating rod/drum
10	swimming
8	hanging/grasping
7	gait
5	inclined plane
4	traverse rods
3	pivoting
2	ambulation times
2	nonspecified, human
Sexual behavior (15)	
12	mating success
5	male performance
2	female receptivity signs
Activity (39)	
23	open field
10	activity wheels
6	photoactomers
3	behavioral sequencing
2	hole board
2	inductance monitors
2	nonspecified human
2	stabilimeter
1	automated plus-maze
1	exploratory
1	home cage
1	motron
1	residential unit
7	some monitoring of activity for ≥ 24-h period
Aggression (6)	
3	shock-induced
1	muricide
1	nonspecified, human
1	tail-rattling
Classical conditioning (2)	
1	nonspecified, human
1	tone-shock pairing
Avoidance (26)	
12	passive
12	2-way active
7	1-way active
2	nonspecified CAR
2	Sidman
2	Y-maze

Table 2.8 (continued)

Operant behavior (17)
- 13 simple schedules
  - 9 FR
  - 7 DRL
  - 4 CRF
  - 4 VI
  - 3 FI
  - 2 punishment
- 9 discrimination procedures
- 6 acquisition/extinction
- 4 multiple schedules
- 3 progressive/titrating schedules
- 2 various nonspecified types
- 1 discrete trial

Maze/instrumental behavior (30)
- 15 water mazes
- 9 discrimination procedures
- 4 T-maze
- 3 neonatal home-cage seeking maze
- 3 nonspecified types
- 3 straight alley
- 3 Y-maze
- 2 Hebb-Williams
- 2 Lashley III
- 7 reversal learning
- 2 delayed responding

Social/group behavior (4)
- 2 competitive situation
- 1 group homing response (4)
- 1 nonspecified
- 1 residential unit

Sensory function (29)
- 4 thresholds
  - 3 auditory
  - 2 taste/olfactory
  - 1 shock
- 3 photophobia
- 22 assessed as part of reflex development
- 9 assessed as part of a discrimination paradigm

Pharmacologic/environmental challenge (12)
- 3 amphetamine
- 2 barbiturate sleep time
- 1 alcohol preference
- 1 audiogenic seizure
- 1 drug discrimination
- 1 LD_{50}
- 1 metrazol seizure
- 1 morphine analgesia
- 1 neonatal CNS lesion
- 1 neonatal 6-OH-dopamine
- 1 nonspecified
- 1 shock stress

Other (7)
- 4 spontaneous alteration
- 3 consummary patterns
- 1 psychological evaluation, children

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[a] Tabulated summary of methods used by those researchers who responded to the questionnaire.

[b] Number of researchers who reported using one or more methods to evaluate behavior falling into each major category.

[c] Number of researchers who reported using the specific method or monitoring the specific behavior.

Source: Buelke-Sam and Kimmel (1979), Table 2, p. 24. Reprinted with permission of the publisher.

2.6.1 General Aspects

No ideal species has been identified with regard to extrapolating experimental results from teratogenic tests in animals to man (NAS 1977; Schardein 1976, 1983; Palmer 1978). That is, there is no one species that fits all the criteria for an ideal test animal as listed by Wilson (1975): (1) absorbs, metabolizes, and eliminates test substances as does man, (2) transmits test substances and their metabolites across the placenta as does man, (3) has embryos and fetuses with developmental and metabolic patterns similar to those of man, (4) is easily bred and has large litters and a short gestation period, (5) is inexpensively maintained under laboratory conditions, and (6) does not bite, scratch, kick, howl, or squeal. The problem of choosing the most appropriate species is further complicated when the test chemical has no record of human administration from which to draw a comparison. Thus, with little scientific data available, the choice of species is often based on availability, economic feasibility, and ease of management (Brown 1963, as reported in Palmer 1978).

Kalter (1968) writes that there are three different types of interspecific and intraspecific susceptibility: (1) an agent teratogenic in some species or groups of animals may be completely or almost completely without teratogenic effect in others, (2) a teratogen may produce similar defects in various species, stocks, or strains of animals but with varying susceptibility (frequency) between one strain and another or between one species and another, and (3) a teratogen may induce one or more abnormalities in some species or groups but have entirely different or only somewhat different effects in others. Kalter indicates that these are not mutually exclusive categories and certain situations may contain features of more than one category. These differences are illustrated in Tables 2.9–2.11 and also in the following sections.

The rat followed by the mouse and the rabbit are the most commonly used laboratory animals for the teratogenic screening of chemical agents (World Health Organization 1967, Palmer 1978). Further substantiation of this fact comes by examination of the holdings of ETIC, whose files contain over 25,000 papers indexed on various aspects of teratogenic testing. Of these, approximately 8350 cite the rat as a test animal, 4640 cite the mouse, 600, the hamster, 1700, the rabbit, and 460, the nonhuman primate. In addition, nearly 9550 papers discuss teratogens and teratogenicity in relation to humans.

2.6.2 Rat

Advantages of using the rat as a test animal for teratogenic testing include (1) short duration of pregnancy (21 to 22 days), (2) high fertility rate, (3) large litters and a relatively good resistance to the toxic effects of most drugs, (4) a fairly good developmental stability, and (5) a low spontaneous rate of major malformations (1 per 1000 fetuses, 0.001%) (Tuchmann-Duplessis 1972, Palmer 1978). The rat is also easily handled

Table 2.9. Species Susceptibility to Drugs

Drug	Dosage (mg/kg) and route	Malformed species (percent incidence)			
		Mouse	Rat	Rabbit	Others
Azathioprine	10 PO[a]	0	0	57	
Cortisone	5 or 10 IM[b]	79	0	50	
Demecolcine	1.5 IP[c] or SC[d]	23	0	3	0 (monkey)
Ethyndiol	1 PO or SC	30	0	0	
Meclizine	125 PO	0	92	0	
Methylprednisolone	1 IM or SC	92	0	0	
Perphenazine	25 PO	100	98	0	

[a] PO—per os (oral).
[b] IM—intramuscular.
[c] IP—intraperitoneal.
[d] SC—subcutaneous.

Source: Adapted from Schardein, 1976, Table 2-8, p. 21. Data collected from several sources. Reprinted with permission of the publisher.

and economically maintained, and test materials can be administered by a wide variety of routes (Palmer 1978). The main limitation of the rat is its poor teratogenic susceptibility to drugs such as cortisone (Thompson and Schweisthal 1969, Hansson and Angervall 1966), azathioprine (Tuchmann-Duplessis and Mercier-Parot 1966), and thalidomide (Somers 1963). Thus, definite conclusions based only on tests with rats could be misleading (Tuchmann-Duplessis 1972).

2.6.3 Mouse

The advantages listed for the use of the rat in testing (Section 2.6.2) also apply to the mouse, with the addition that the mouse is even more economical, has a shorter gestation period (18 to 19 days), has a wide variety of defined inbred strains for special studies, and is more susceptible to some teratogens (Tuchmann-Duplessis 1972, Palmer 1978). Disadvantages include: (1) small size of the fetus and consequent difficulty in examination of malformations; (2) arrangement of the malformations in clusters, which creates difficulty in assessment; (3) higher spontaneous malformation rate than the rat (0.5% in the Swiss albino colony of Tuchmann-Duplessis); and (4) resorption rates much higher than in the rat, necessitating a large control group (Tuchmann-Duplessis 1972, Palmer

Table 2.10. Thalidomide Teratogenesis in Primates

Species	Teratogenic dose (mg/kg, oral route)	Gestation days treated	Defect
Man	0.5 to 1.0	20 to 36	Limbs (80%), ear (20%)
Cynomolgous monkey	10	22 to 32	Limbs (67%), teratomas (33%)
Baboon	5	18 to 44	Limbs and tail (40%)
Rhesus monkey	12 to 19	24 to 26, 27, or 30	Limbs (100%)
Bushbaby	20	16 to 30	
Japanese monkey	20	24 to 26	Limbs (100%), tail (17%), central nervous system (17%)
Stump-tailed monkey	5 to 10	24 to 30	Limbs (100%), tail (20%)
Marmoset	45	25 to 35	Limbs, ear, and jaw (100%)
Bonnet monkey	5 to 30	24 to 29 or 41 to 44	Limbs (45%), visceral (52%)

Source: Adapted from Schardein, 1976, Table 2-11, p. 25. Data collected from several sources. Reprinted with permission of the publisher.

1978). The mouse is also known to respond differently than the rat to some drugs such as cortisone.

With regard to the human teratogen thalidomide (see also Section 2.6.8.3), mice embryos are relatively insensitive, as is evident in a study by Somers (1963) in which oral administration of doses up to 400 mg/kg throughout pregnancy did not reduce the number of newborn mice or their ability to survive to weaning. Even when the doses were increased to 4000 mg/kg only a small increase in resorption sites was observed.

2.6.4 Rabbit

The rabbit was the first laboratory animal shown to be susceptible to the human teratogen thalidomide (Somers 1962) and has since been considered by many biologists to be one of the most favorable animals for teratogenic studies (Tuchmann-Duplessis 1972). The susceptibility of the rabbit to thalidomide was also reported by Somers (1963), who produced limb defects in the offspring by administering 150 mg/kg to pregnant rabbits on days 8 to 16 of gestation, and by Shepard (1976), who states that limb defects in New Zealand rabbits were observed after administration of 250 mg/kg on days 8 to 10 of pregnancy.

Table 2.11. Comparative Teratogenicity of Thalidomide

Organ system affected	Human	Nonhuman primate	Rabbit	Rat	Mouse
Limbs	X	X	X	X (low%)	X (low%)
Ears	X	X			
Eyes	X	X			
Cardiovascular	X	X	X	X	
Urinary	X	X	X		
Genital	X	X	X		
Gastrointestinal	X	X	X		
Pulmonary	X				
Prenatal and/or postnatal death	X	X	X	X	X

Source: Hendrickx et al. 1983, Table II, p. 153. Reprinted with permission of the publisher.

Advantages of using the rabbit include the larger size of the rabbit fetus compared to the rat or mouse (Palmer 1978); the ability of the rabbit to show a variety of spontaneous malformations, which suggests that this species would be more susceptible than some other species to a variety of teratogenic actions (Palmer 1968, as reported in Palmer 1978); and, for comparison purposes, the rabbit's physiological difference from the rat and the mouse (Tuchmann-Duplessis 1972). The necessity of having large control groups, a higher spontaneous malformation rate (1.7% for a 5-year period) (Tuchmann-Duplessis 1972), and the rabbit's dependence on gut flora for nutrition, which prevents the testing of antibiotics and makes incorporation into the diet a hazardous and inaccurate means of administering test compounds (Palmer 1978), are some disadvantages of using the rabbit as a model for testing compounds for teratogenicity. More practical disadvantages are problems associated with handling and intubation.

2.6.5 Hamster

Palmer (1978) reports that the hamster is mainly a competitor for the mouse in animal studies; it is almost as economical to maintain and is reputed to be more stable genetically. The hamster has several advantages that make it a potentially valuable animal for developmental and teratogenic studies: unique reproductive features such as large litter size and short gestation period (16 days) (Ferm 1967). Palmer (1978), however, lists several disadvantages of using the hamster: (1) aggressive behavior generally necessitates individual caging; (2) achieving precisely timed mating, although not difficult, is not as convenient as with the mouse;

(3) intravenous injection is more difficult than in the mouse [Ferm (1967) does demonstrate a method requiring anesthesia whereby the injection is made utilizing the lingual vein]; and (4) as with the rabbit, the hamster's gut flora renders it susceptible to change in the diet and to antibiotics. Also, the hamster has a poor historical data base. Tuchmann-Duplessis (1972) concludes that from available data the hamster does not have a definite advantage as a test animal in the teratogenic screening of drugs.

2.6.6 Nonhuman Primates

Much has been written concerning the use of nonhuman primates as animal models in the teratological testing of chemicals (Wilson et al. 1968; Wilson 1969, 1971a, 1971b, 1978; Hendrickx 1972; Poswillo et al. 1972; Tanimura 1972; Siddall 1978). The rhesus monkey (*Macaca mulatta*) is the most popular nonhuman primate test species according to Wilson (1978), but teratological studies have been performed using other nonhuman primates such as the cotton-eared marmoset (Poswillo et al. 1972; Siddall 1978) and the baboon (Hendrickx 1972).

One of the primary advantages of using nonhuman primates in the teratological screening of chemicals is their similarity in many respects to man. Wilson (1978) reports that: (1) in some instances the metabolism of drugs in man approximates that of other higher primates; (2) the reproductive physiology of Old World monkeys (rhesus monkeys and baboons) closely resembles that of man, particularly the menstrual cycle, spermatogenesis, and parturition; (3) the placental structure of man and nonhuman primates appears to be quite similar, although the similarities of function during the critical periods of gestation have yet to be fully established; (4) the anatomical and temporal aspects of embryonic development between the higher nonhuman primates and man are strikingly similar; and (5) similarity in response to thalidomide is well established [(the comparative response to other agents, however, has been variable (Section 2.6.8)].

Similarly, Leone (1977) states in an excellent review of the developmental stages in mammals used for teratogenetic tests that "Significant differences are detectable between rodents and primates in the early stages of development, which especially affect the structure and functions of the embryonic membranes and consequently influence the teratogenic process." In his opinion, the morphological and comparative aspects of development deserve as much attention as do other parameters (i.e., metabolic pathways, dose threshold, etc.).

In a discussion favoring greater diversity in test species used for teratogenicity testing, Wilson (1975a) states that rodents and rabbit embryos are dependent during the initial days of organogenesis on the inverted yolk-sac placenta, which is structurally and functionally different from the chorioallantoic placenta of other mammals (e.g., primates) at corresponding times in development.

Wilson (1978) cites scarcity; difficulties in handling, especially the unpredictability of behavior and general susceptibility to infections; and low fecundity as disadvantages to using nonhuman primates for teratological testing of drugs. Two other disadvantages of nonhuman primates are the expense (high cost usually necessitates a test group size that is not adequate) and the long gestation period.

It should be mentioned that prosimians, and to a certain extent New World monkeys such as marmosets, have not been used extensively for teratological evaluations of chemicals. The greater bush baby (*Galago crassicaudata*), a prosimian, was found by Hendrickx (1972) to give a negative response to thalidomide and has since received little attention. The marmoset has shown similar responses as man to thalidomide (Poswillo et al. 1972, Siddall 1978), but the fact that marmosets do not menstruate causes difficulty in following the reproductive cycle and possibly accounts for their sparse use (Wilson 1978). Because of the advantages afforded by their smaller size, however, Wilson (1978) recommends further testing of the suitability of New World monkeys and prosimians as animal models in teratology.

2.6.7 Other Species

Other animals such as dogs, cats, and swine have been used for teratological testing but not to the extent of the previously mentioned species.

2.6.7.1 Dog

Palmer (1978) writes that the primary advantage of the dog is its concurrent use as a nonrodent species for other toxicity tests that may provide further information of the test material such as pharmacokinetics. Earl et al. (1973) tested the beagle with the known human teratogens thalidomide and aminopterin as well as with methyl mercuric chloride, hydroxyzine, and hydroxyurea. They found that the dog (1) responds to known human teratogens, but not in a classical manner, (2) does not appear to be as sensitive an indicator of the above-mentioned compounds as some primates, and (3) offers little advantage over other laboratory animals. In addition, the frequency of estrus (twice a year), a high cost of maintenance, and a poor historical data base are also disadvantages.

2.6.7.2 Cat

The cat has been used infrequently in teratology studies for such reasons as the difficulty in exact determination of the time of ovulation and the time required to establish a stable colony (Palmer 1968). In addition, the cat has a poor historical data base from which to make comparisons. Khera (1975) did show that thalidomide (10 to 480 mg/kg per day) induced a wide variety of fetal cardiovascular defects including ventricular septal defect, malpositioned great vessels, and narrowed left ventricular chamber with hypertrophied walls.

Other research by Khera and coworkers (Khera and Iverson 1978, Iverson et al. 1980), however, demonstrated that the cat is not as susceptible to the teratogenic effects of some chemicals as are other animal species. Khera and Iverson (1978) administered ethylenethiourea (a degradation product of the ethylenebisdithiocarbamate group of fungicides) orally in single daily doses of 0, 5, 10, 30, 60, or 120 mg/kg to 14, 9, 7, 8, 7, and 10 female cats, respectively, beginning on day 16 of gestation and continuing through pregnancy unless maternal toxicity was exhibited. Although ethylenethiourea proved to be toxic to pregnant cats (several cats died or were killed in a moribund condition in the 30-, 60- and 120-mg/kg dose groups) and had caused malformations in some of the dead fetuses taken from those cats killed in the moribund state, no clear evidence for teratogenicity of ethylenethiourea in live fetuses was seen. This result is in contrast to the ethylenethiourea-induced teratogenesis observed in rats (Khera 1973; Khera and Tryphonas 1977, as reported by Khera and Iverson 1978). In a follow-up study, Iverson et al. (1980) suggest that the reason for the absence of teratogenic effects in the cat was the ability of the cat to extensively metabolize ethylenethiourea to its S-methyl derivative; examination of rat urine showed no evidence of the S-methyl derivative.

2.6.7.3 Pig

Tuchmann-Duplessis (1972) and Palmer (1978) list several advantages of using the pig: (1) it is easily available and highly prolific compared to other nonrodent species, (2) its embryology and genetics are well known, (3) pregnancy can be obtained year round, and (4) incorporation of the test material into the diet is easily and accurately accomplished because of the pig's voracious eating habits. These same authors cite disadvantages of using the pig as the large amounts of the test material required due to the size of the pig and the excessive floor space required. Earl et al. (1973) observed malformed offspring in miniature swine that had been exposed to thalidomide, hydroxyurea, and aminopterin and conclude that, although the reaction to thalidomide was not the classical response, miniature swine offer certain advantages such as low incidence of spontaneous malformations. These researchers recognize that more data are necessary before the role of the pig in teratogenic studies can be determined.

2.6.8 Results of Testing Some Human Teratogenic Chemicals in Animal Models

Three known human teratogens, aminopterin, methotrexate, and thalidomide, will be used to compare the teratogenic response between humans and several animal species; the data are summarized in Tables 2.2, 2.3, and 2.4.

2.6.8.1 Aminopterin

In addition to man, the folic acid antagonist aminopterin (Table 2.3) induced malformations in the mouse but not in the rat, where embryolethality and resorptions were observed, or in the rhesus (*Macaca mulatta*) and cynomolgus (*Macaca irus*) monkeys. The one reference concerning aminopterin toxicity in the rabbit reports no injury to a six-day-old blastocyst, but the assumption from these data that no injury would result to the fetus probably would not be reliable.

2.6.8.2 Methotrexate

Methotrexate, also a folic acid antagonist and a methyl derivative of aminopterin, is teratogenic to humans, mice, rats, and rabbits but apparently not to monkeys, although abortions were induced (Table 2.4). The data in Table 2.4 indicate that (1) teratogenic defects in the man, mouse, rat, and rabbit are somewhat similar, especially with regard to rib and limb abnormalities; (2) the rat shows a teratogenic response to methotrexate but not to aminopterin (Table 2.3); and (3) the monkey seems to be resistant to the teratogenic action of both methotrexate and aminopterin (Table 2.3). Skalko and Gold (1974) point out, however, that no teratogenic effects are produced in mice at doses that are teratogenic in humans, rats, and rabbits and abortifacient in rhesus monkeys (0.3 to 10 mg/kg), thus suggesting that the mouse embryo is more resistant to the embryotoxic effects of methotrexate than any other mammalian species yet studied. The minimum dose required to produce teratogenicity in the mouse was 25 mg/kg and is the same as that necessary for aminopterin to be teratogenic (Table 2.3). In rabbits, methotrexate-induced abnormalities increase as the dose increases, a fact that is related to metabolism by the maternal system (Jordan 1974). Jordan discovered that when methotrexate is present in low concentrations, it is rapidly converted by the maternal enzyme aldehyde oxidase to a relatively inactive metabolite, 7-hydroxymethotrexate; at higher levels of methotrexate, the enzyme becomes saturated and, consequently, more unmetabolized drug reaches the embryo.

2.6.8.3 Thalidomide

Table 2.2 indicates that human, monkey, and rabbit embryos are highly susceptible to thalidomide but that mouse, rat, and hamster embryos are not nearly as sensitive and show apparent strain differences. In the study by Parkhie and Webb (1983) thalidomide teratogenicity was demonstrated in Wistar rats by the administration of single intravenous doses. This is in contrast to the data of Somers (1963) (see Table 2.2), who, however, administered the compound orally. Parkhie and Webb state that the intravenous administration of thalidomide may circumvent the factors involved in its loss of teratogenicity when given orally.

Although both the monkey and the rabbit embryos exhibit malformations similar to those observed in the human fetus, especially with regard to limb defects, Delahunt (1965) concludes that the thalidomide-induced abnormalities in man are much more similar to those in the monkey than they are to those in the rabbit. Although Drobeck et al. (1965) do not disagree with Delahunt, they do believe that the results observed in the rabbit with thalidomide generally confirm what has been reported in humans. They continue by stating that micromelia and phocomelia in the human cases have been so popularized as to overshadow the wide range of effects that has actually been observed including developmental failure of visceral organs such as the kidneys and adrenals as well as developmental failure in bony structures. According to Drobeck and coworkers, these effects are well characterized in the rabbit.

Detailed examination of the data on rats, hamsters, and mice in Table 2.2 illustrates the apparent strain differences in susceptibility to thalidomide. Fratta et al. (1965) found no teratogenicity in mice strains CF_1, ICR, C_{57}, CBA, and SJL; DiPaolo et al. (1964) induced malformations in strain A, C_3H, and Swiss. Similar results are seen in hamsters, where inbred lines are susceptible and random bred lines are not, and in rats, where the Sprague-Dawley and Holtzmann (descended from Sprague-Dawley) strains and one unidentified strain produced malformed young, whereas the Wistar (by oral administration), Charles River, Long Evans, and Dunning Fischer strains did not.

Although many explanations have been offered for species or strain susceptibility to the teratogenicity of thalidomide, the issue has not been resolved. Gordon et al. (1981) postulated that thalidomide is metabolized to a toxic electrophilic intermediate that is not produced in vitro by liver microsomes from Sprague-Dawley rats, which, they state are not a sensitive species. This intermediate was produced by hepatic preparations from rabbits, monkeys, and humans, species regarded by Gordon et al. (1981) as sensitive. However, Table 2.2 shows that some strains of rats, including Sprague-Dawley, have demonstrated the teratogenicity of thalidomide.

Vaisman et al. (1983) have postulated that the mechanism of the teratogenic action of thalidomide is the induction of a deficit of ascorbic acid in species such as nonhuman primates and man that do not have the ability to synthesize this vitamin. In their tests with the guinea pig, which also cannot synthesize ascorbic acid, thalidomide was shown to induce a deficit of ascorbic acid in both adults and fetuses. Their premise is based on the fact that a deficiency of ascorbic acid leads to marked inhibition of collagen synthesis (Matusis 1975, as reported in Vaisman et al. 1983) which may damage the anlagen of the limbs. However, teratogenicity has been demonstrated in rabbits (including limb malformations) and, although perhaps not as convincingly, in rats, mice, and hamsters (see Table 2.2), which can synthesize ascorbic acid.

Thus, as stated by Fabro (1981), "We must accept the facts that a clear understanding of the mechanism by which thalidomide exerts its teratogenic effects escapes us after almost two decades of intense scrutiny and that the teratogen thalidomide still remains an embryologic enigma."

2.7 RECENT APPROACHES IN TERATOLOGY TESTING

In recent years alternative approaches to the standard methods of testing agents for teratogenicity have been investigated, primarily due to economic and practical considerations. Most, although not all, of the recently proposed methods have been in vitro test procedures. An investigation of the current holdings of the ETIC concerning mammalian studies reveals 889 indexed reference citations pertaining to various aspects of in vitro teratogenicity. Analysis of these with respect to species indicates that 322 are associated with rats, 409 with mice, 14 with hamsters, 48 with rabbits, 1 with monkeys, and 105 with humans. The total is small compared to the approximately 25,000 citations recorded by ETIC for in vivo testing, but, as many of the citations for in vitro testing are within the last 5 to 8 years, the current interest in alternatives to whole mammal testing is evident.

2.7.1 In Vivo Methods

An alternative to current embryo toxicity screening techniques was proposed by Marhan and Jelinek (1979) using a caudal morphogenetic system. In this proposed teratology screen, which the authors describe as a compromise approach, the test substance is administered to rats on days 10 and 11 of pregnancy, and the effects on the activity of the caudal morphogenetic system are determined by measurements of those parts of the trunk arising through activity of that system of 13-day-old embryos. The authors concluded that "the caudal morphogenetic system of rat embryos reflected some of the changes induced in the maternal-embryonic complex."

Chernoff and Kavlock (1982) have proposed a teratology screen that differs from previously employed in vivo testing methods in that it does not require the labor-intensive examination of fetuses for soft-tissue and skeletal anomalies. Rather, the dams (CD-1 mice were used in the 1982 study) are allowed to give birth, and determinations of teratogenic potential are made on the basis of litter size and weight on postpartum days 1 and 3 as compared with concurrent controls. Also, because the intent is priority-setting and not testing, only one dose level (at or near the maximally toxic dose) is used, in comparison with at least three levels in conventional teratology test methods.

In the 1982 study, Chernoff and Kavlock tested 28 compounds of known teratogenic potential—15 that were teratogenic by standard test criteria did exhibit some form of developmental toxicity; 2 chemicals known to produce only reduced weight caused reduced weight (endrin) or reduced litter size (sodium selenite) in the proposed assay; 2 compounds which in standard tests caused only an increased incidence of supernumerary ribs produced no effect in the proposed screen; and of 9 compounds for which no effects had been reported in conventional test procedures, 6 produced no effects in the screen and 3 produced positive effects

(2 compounds caused reduction in litter weight and 1 caused reduced litter size).

Chernoff and Kavlock (1982) state that their teratology screen can be used to divide chemicals into three categories: (1) those that induce perinatal death should be tested as rapidly as possible, (2) those that induce only perinatal weight changes would be given a lower testing priority, and (3) those producing no effect would be given the lowest priority. In October 1984, the EPA released guidelines for the use of this preliminary developmental toxicity screen. Personal communication with Dr. Kavlock has indicated that approximately 10 to 12 laboratories are currently using this proposed method. Results of using this teratology screen in four of these laboratories have been reported by Schuler et al. (1984). Fifteen glycol ethers were tested using Charles River CD-1 mice (no two laboratories tested the same chemical). Schular et al. provided results from conventional testing for five of these. Of the five, three were known teratogens, and a correlation with results of the proposed screen was seen in that these three compounds were designated either in a high priority or middle to high priority group. Two of the five compounds showed no reproductive toxicity in conventional tests; one was assigned to the middle priority group and the other to the low priority group in the in vivo screen.

2.7.2 In Vitro Methods

Over the last few years several in vitro methods for screening chemicals for teratogenicity have been proposed. Interest in testing for teratogenic chemicals in vitro was evident as early as 1975, when the International Conference on Tests of Teratogenicity In Vitro was held (Kimmel et al. 1982b). In August 1981 the consensus workshop on In Vitro Teratogenesis Testing was convened in Arkadelphia, Arkansas, and the proceedings were published in the *Journal of Teratogenesis, Carcinogenesis, and Mutagenesis* (Vol. 2, 1982). Much of the information presented below is taken from those proceedings. As defined at the workshop and used in this section, the term in vitro refers to any system that uses test subjects other than the intact pregnant mammal.

2.7.2.1 Whole mammalian embryo culture

Sadler et al. (1982) tested the potential teratogenicity of several compounds using a whole mouse embryo procedure. Their approach was to administer the test compound at the 4- and 5-somite stages, which according to them showed survival and a high percentage of malformations. They found that early stages (primitive streak to 0-1 somites) were often severely affected and in some cases failed to survive. Sadler and coworkers showed that glucose, ketone bodies, vitamin A, and hyperthermia produced malformations (neural tube and/or cardiac effects) in vivo. Although stating that the whole-embryo culture system may be useful as a screening technique for potentially teratogenic substances, they recognize that unanswered questions, such as the appropriate exposure time, point to the need for further investigation before standardized protocols can be developed.

The reader is referred to a paper by Kochhar (1975) for a description of early attempts to culture whole mammalian embryos.

2.7.2.2 Embryonic limb bud organ culture

As described by Kochhar (1982), this method involves the removal of limb buds from mouse embryos of known ages (usually of the 12th day of gestation). Limb buds are placed on top of ultrathin Millipore or Nucleopore Filters and incubated in a nutrient medium in the presence or absence of test chemicals. End points that may be monitored include cell proliferation, differential growth, morphogenetic cell death, size and shape of limb parts, chondrogenesis, and collagen or proteoglycan biosynthesis. Kochhar stated that validation was still in the early stages; to achieve full potential the system should be combined with an efficient drug-metabolizing preparation.

2.7.2.3 Avian embryonic cells

Wilk et al. (1980) describe two in vitro systems using chick embryos, one using neural crest cells obtained from very early chick embryos and the other, limb bud mesenchyme cells obtained at a later stage of development. Tests were performed both with and without S-9 added for metabolic activation. The S-9 fraction was toxic to cells when added directly to the medium, and thus the S-9 plus a NADPH-generating system and test compound was combined in autoclaved dialysis bags and added to the cultures. A combination of both the neural crest and limb mesenchyme system correctly predicted the teratogenicity of the known teratogens tested with the exception of thalidomide. Isoniazid and glutethimide, which are not teratogenic in vivo, were also inactive in the Wilk et al. system. Wilk et al. (1980) state that a modification of their system by incorporating the use of postmitochondrial fractions from placental and fetal tissues may improve its sensitivity and enable the detection of the teratogenic activity of thalidomide.

2.7.2.4 Ascites tumor cell assay

An in vitro teratogenicity assay that identifies teratogens by their ability to inhibit the attachment of ascitic mouse ovarian tumor cells to plastic surfaces coated with concanavalin A has been proposed by Braun et al. (1982). This system correctly identified 60 known teratogens, including thalidomide, but failed to predict the teratogenicity of 14 known teratogens, including X irradiation and methotrexate. With respect to predicting nonteratogenicity, 21 nonteratogens were correctly identified but 7 compounds were false positives. Recognizing the limitations of any single in vitro system, the authors state that "attachment inhibition in concert with other, complementary, in vitro assay systems can become a useful method for all assessment of the teratogenic potential of environmental agents." [According to the National Toxicology Program's annual plan for 1984 (NTP 1984), a complementary in vitro test measuring the chemical inhibition of growth potential of human embryonic mesenchymal cells is currently being conducted by two separate laboratories.]

2.7.2.5 *Drosophila* embryo cell assay

Bournias-Vardiabasis and Teplitz (1982) report the development of an in vitro assay that detects teratogens by adding them to primary cultures of embryonic *Drosophila* cells and analyzing the degree of change in cell differentiation and tissue formation. In validation tests of over 100 commonly used industrial chemicals, drugs, and food additives, only two false negatives (ethyl alcohol and cyclophosphamide) and two false positives (p-aminobenzoic acid and dimethylamino antipyrine) were observed. When the serum of animals fed the test agent is added to the differentiating culture, metabolic products of ingested compounds can be tested for teratogenicity. The authors note that further testing, validation, and incorporation of a metabolic activation system are needed.

2.7.2.6 Poxvirus morphogenesis

Because the poxvirus has a rapid, simple morphogenetic pathway that is dependent upon cell proliferation, the effect on the growth of poxvirons in cell culture can be used as an indicator of teratogenic potential (Keller and Smith 1982). In this assay, vaccinia WR-infected BSC 40 monolayers are exposed to the test agent for 24 h; the number of functional virons is then determined by plaque assay. In tests with 42 known teratogens, Keller and Smith observed that 33 inhibited the number of virons produced and 3 stimulated the production virons and were thus considered teratogenic by this assay. Of nine nonteratogens tested, all but one (ascorbic acid) were negative in the poxvirus assay. Thus six teratogens were false negatives and one was a false positive.

2.7.2.7 Neuroblastoma cells

Mummery et al. (1984) investigated the ability of 39 teratogenic and 18 nonteratogenic compounds to interfere with normal growth and differentiation of murine neuroblastoma cells (clone NIE-115). This system correctly predicted the teratogenicity of 35 of the 39 and the nonteratogenicity of 14 of the 18. The authors state that substances such as thalidomide that require metabolic activation for teratogenicity are unlikely to be detected by this system.

2.7.2.8 *Hydra attenuata* system

Johnson et al. (1982) describe a teratogenic screen that uses the freshwater *Hydra attenuata*. The first of a two-part experiment involves exposing adult hydra to a test substance over a broad range of concentrations and determining the minimal toxic concentration to within one-tenth log. In the second experiment, cells of dissociated hydra are manipulated into a configuration that permits them to achieve the developmental events characteristic of any embryo and undergo total whole-body regeneration; these cells are exposed during a 4-day ontogenesis period to the test substance in the same manner as the adult, and the minimally developmental toxic concentration is determined to one-tenth log. Then, the ratio of the adult (A) to the developmentally (D) toxic concentration is calculated. A small A/D ratio indicates that the agent affects adults and developmental events at generally the same dosage and, provided an adult toxic dose level is not attained, the embryo will not be at risk. A large A/D ratio indicates the agent is a primary developmental hazard with the conceptus as the most vulnerable target, and at risk even at sub-adult toxic exposure levels. This system is also described by Johnson and Gabel (1982) and by Johnson (1984).

The *Hydra attenuata* system has been validated by comparing the A/D ratios for chemicals tested using this system with the A/D ratios for chemicals tested using conventional developmental toxicity test procedures. Data supplied by Dr. Johnson of comparison of the A/D ratios of approximately 40 test substances indicate a good correlation in most instances. The A/D ratios of mammals and hydra seldom varied by as much as a factor of 2. One exception was 6-aminonicotinamide with A/D ratios of over 4 for mammals and 26 for hydra. Personal communication with Dr. Johnson indicates that the use of the bioactivation in the hydra system is currently being investigated under the aegis of the March of Dimes.

2.7.2.9 Planarian assay

Best and Morita (1982) describe the results of experiments using freshwater planarians as indicator organisms in a teratogenicity assay. Either surgical fragments of planarians that undergo regeneration or intact planarians can be used as test organisms. When fragments are used, distortion of the regeneration is the measure of teratogenicity, and with intact planarians the development of morphologic or other observable abnormalities is the indicator. Citing the results of testing several chemicals for teratogenicity, Best and Morita state that "both regeneration of surgical fragments and aberrant remodeling of whole planarians model important features of embryogenesis and are potentially useful for assaying teratogens." The authors suggest that the planarian system might also be useful in screening for chemicals that cause behavioral teratogenicity.

2.7.2.10 Frog embryo assay

In this method described by Dumont et al. (1982) and Dumont and Epler (1984), early frog embryos are exposed for 96 h to concentration ranges of suspect agents. The following end points may be observed: LC_{50}, EC_{50} (concentration required to produce 50% terata among survivors), growth and developmental stages attained, anatomical abnormalities, motility (behavior), and pigmentation. Personal communication with Dr. Dumont indicates that 7 of 41 compounds (17%) tested were false negatives and 5 of 41 (12%) were false positives.

2.8 CONCLUSIONS AND RECOMMENDATIONS FOR FURTHER RESEARCH

2.8.1 Conclusions

2.8.1.1 General experimental considerations

1. At least three dose levels of a chemical should be tested. The low dose should allow normal development, the intermediate dose should show teratogenicity if the chemical tested is a teratogen, and the high dose should produce either maternal toxicity, embryotoxicity, or fetotoxicity.
2. Positive controls cannot provide certainty that the test chemical is not teratogenic if tests indicate no teratogenicity.
3. For each chemical at least two species should be tested for teratogenicity.
4. The number of pregnant test animals per group must be sufficient to permit reliable statistical analysis. A minimum of 20 pregnant animals of a rodent species and 12 pregnant rabbits has been recommended by several regulatory groups.
5. The administration route should be oral intubation unless the physical and chemical properties of the chemical or human use conditions dictate otherwise.
6. All fetuses should be examined for external malformations. Approximately one-third to one-half of the fetuses of rats, mice, and hamsters should be examined for skeletal defects and the remainder for soft tissue malformation. All rabbit fetuses should be examined for both skeletal and soft tissue anomalies.

2.8.1.2 Structure-activity relationships

Although selected teratogenicity studies seem to illustrate certain structure-activity relationships, generalizations regarding the prediction of human risks from chemical structure are not yet feasible. This conclusion is supported by the statement of Zimmerman (1975) that "Prediction of teratogenesis by chemical structure will have to await a future time" and of that by Schumacher (1975) that "Structure-activity studies, as performed until now, will not, we feel, provide the needed information and are therefore of no predictive value with regard to teratogenicity in animals, much less in humans."

2.8.1.3 Teratogenesis and time of administration

When a chemical is tested for teratogenicity, observations for behavioral as well as physical defects should be performed. Although it is generally recognized that organogenesis is the most sensitive prenatal developmental period to the malforming action of teratogenic chemicals, the possibility of a test chemical inducing behavioral teratogenic effects when administered in each of the three trimesters (and even pregestationally, see Section 2.5) must be considered.

2.8.1.4 Behavioral teratogenesis

After a slow start, interest in behavioral teratogenicity increased during the early 1970s, and today it is a widely recognized and respected science. Although some countries (the United States excluded) have proposed guidelines for the testing of agents for behavioral teratogenicity, standardization of test methodologies is still needed.

2.8.1.5 Species comparisons

There is no ideal animal model for use in teratological research. The monkey (primarily the higher primates) has received considerable attention in recent years, especially since the thalidomide syndrome appears to be mimicked to a high degree in the monkey. This animal offers the advantage of having a chorioallantoic placenta as does man, in contrast to the inverted yolk-sac placenta of rodents and rabbits, and having other anatomical and physiological similarities to man. Yet, studies using the folic acid antagonists methotrexate and aminopterin, known human teratogens, have been unable to demonstrate any significant teratogenicity in the monkey, although abortions have been induced. However, if a compound whose embryotoxicity is being tested was shown to have abortifacient action but not necessarily teratogenic potential (inducing malformed offspring), the use of the compound would probably be restricted for women of child-bearing age. Thus, the decision on potential use of this compound by pregnant women would probably be the same whether it induced malformations or abortions. Using the criteria of embryotoxicity (embryolethality, malformations, or growth retardation), Wilson (1971a) has shown that the effects induced by several agents in man and in the rhesus monkey (chemical and viral) are comparable. Wilson acknowledges that before the rhesus monkey can be considered more reliable than other laboratory animals for anticipating embryotoxicity in man, more detailed comparative information on drug metabolism and distribution between laboratory animals must be obtained. However, even if the rhesus monkey is shown to be more reliable than other laboratory animals for predicting embryotoxicity in man, the expense will likely prohibit serious consideration as a routine test animal for embryotoxicity.

Rats, mice, and rabbits have been and will probably continue to be used more frequently than the monkey as laboratory test animals primarily due to availability, ease of handling, and economy. Rats and mice have been the most commonly used, but use of the rabbit has increased since the early 1960s because the rabbit was the first laboratory animal shown to be susceptible to thalidomide teratogenicity.

All chemicals do not require the same degree of teratogenicity testing. Compounds for which human exposure, especially to pregnant women, is considered a probability should receive more intensive teratological testing than those chemicals for which human exposure is a remote possibility. In the same vein, Wilson (1978) believes that despite the limited availability of higher primates, testing with macaques and baboons is essential for those drugs needed for therapeutic purposes during human pregnancy and, perhaps, for the environmental chemicals to which women may be inadvertently heavily exposed prior to the diagnosis of pregnancy. Wilson continues by stating that potential teratogenic effects to the central nervous system can be adequately tested only in those higher primates that have, at least roughly, a range of mental and nervous activities comparable to man.

2.8.1.6 Recent approaches in teratology testing

Investigations of practical alternatives to standard in vivo testing methods for teratogenicity screening are needed because of the cost and labor-intensive efforts. Although a few recent approaches have used in vivo procedures, most have used systems other than the whole mammal. At present, although validations of some of these in vitro methods are still being conducted, no single test can be considered as a screen; a battery of tests is more appropriate. For further insights into in vitro screening of agents for teratogenicity the reader is referred to the overview of the 1981 proceedings of the consensus workshop on In Vitro Teratogenesis Testing (Kimmel et al. 1982b).

2.8.2 Recommendations for Further Research

The following are identified as possible research areas for the teratogenicity testing of chemicals:

- An extensive analysis, using existing data, of the teratogenic action of a select group of chemicals representing different chemical classes. Such an analysis should enhance our understanding of species specificity, mechanisms of action, and structure-activity relationships.

- Further investigation of postnatal behavioral changes as they relate to pregestational and gestational chemical exposure.
- Further research directed toward standardization of the methodology for screening chemicals for behavioral teratogenicity.
- Further research on variations on present in vivo test procedures, especially with regard to economy and simplicity.
- Continued validation of existing in vitro methods for screening compounds for teratogenicity as well as further research on the identification of additional in vitro test systems.
- In conjunction with the above, determination of an acceptable battery of tests for in vitro screening of compounds for teratogenicity.

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3. IMMUNOTOXICOLOGY

Mary Lou Daugherty

3.1 INTRODUCTION

The science of immunology, once limited to studies of host-defense mechanisms against bacterial, fungal, viral, protozoal, and helminth diseases, has expanded into a complex but well-studied discipline that includes allergy, tumor immunity, host-graft reactions, tolerance, and the immune mechanisms involved in the pathogenesis of various occupational disorders of the lung and skin.

These protective responses of the body against foreign entities (or antigens) are carried out by the immune system, a complex and widespread organization of organs and cells. Immune responses are of two types: nonspecific (or innate) and specific (or acquired) (De Bruin 1976).

Nonspecific immunity is characterized by mechanisms for the disposal of foreign and potentially harmful macromolecules, microorganisms, or metazoa which do not involve the recognition of antigen or the mounting of an immune response. Nonspecific immunity is generalized and includes the activities of phagocytic cells, certain microbial inhibitors (lysozymes), the interferon system, the complement system, and the kinin system.

Specific immunity is characterized by (1) the highly specific recognition of and response to antigen by lymphocytes and (2) the induction of immunological memory in long-lived lymphocytes (memory cells), which are then capable of producing an enhanced immune response upon second contact with an antigen (Luster et al. 1982b). Specific immunity includes: cell-mediated immunity, humoral immunity, and immunological tolerance. The major organs of the specific immune system in man are the thymus and bursa-equivalent, which are the primary lymphoid tissues, and the lymph nodes, the spleen, the bone marrow, the gut-associated lymphoid tissue (GALT) (e.g. Peyer's patches), and the bronchus-associated lymphoid tissue (BALT), which are the peripheral lymphoid tissues (Luster et al. 1982b). The major cells are the circulating lymphocytes. These cells play a central role in both cellular and humoral immunity and and can be divided into two distinct populations, T-cells and B-cells, with different functions and properties (Vos 1977). Also important are the macrophages, both fixed and free, which are involved in and seem to be necessary for the initiation and the effector phases of humoral and cell-mediated immune reactions (Faith et al. 1980). Unlike the lymphocytes, however, the macrophages do not clonally divide and are not antigen specific. Other cell types, such as monocytes, granulocytes, mast cells, and reticuloendothelial cells, facilitate in various ways the expression of the immune response even though they are not equipped ab initio to recognize and interact with antigen or to participate in the induction of the immune response (Miller 1975).

Because the immune system plays a critical role in the protection of man against infectious and neoplastic disease, dysfunction of the system can have serious health consequences (Gardner et al. 1979); it therefore becomes important to be able to identify immunotoxic agents that may be released into the workplace or environment.

Although the concept of immunotoxicology is relatively new, it has been known for some time that certain drugs can cause suppression of the immune system.

In recent years, chemicals other than those used clinically for immune suppression have been recognized as agents capable of inducing alterations in the immune response. These chemicals are of environmental concern and include polychlorinated biphenyls, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, hexachlorobenzene, polybrominated biphenyl, chlorinated dibenzofuran, NO_2, SO_2, benzo[a]pyrene, tobacco smoke, heavy metals, organotins, butylated hydroxytoluene, ortho-phenyl phenol, and pesticides such as DDT, Dieldrin, organochlorines, and organophosphates (Vos 1977, Moore 1979, Silkworth and Loose 1981, Luster et al. 1982b). Information from a variety of human studies (reviewed by Holt and Keast 1977) indicates that increased prevalence of infections, and perhaps neoplastic diseases, resulting from chronic exposure to air contaminants is associated with impaired immunological control mechanisms. These human data are supported by the results of animal studies that demonstrate the impairment of host resistance by atmospheric contaminants such as pesticides, metals, and cigarette smoke [reviewed by Koller (1979a, 1979b), Treagan (1975), Vos (1977), Moore and Faith (1976), Holt and Keast (1977), and Faith et al. (1980)]. In addition, Loose et al. (1977, 1978, 1979) demonstrated that polychlorinated biphenyl and hexachlorobenzene were profoundly immunosuppressive at clinically subtoxic levels and suggested that the evaluation of immune parameters may prove to be a sensitive indicator of general toxicity.

Procedures for testing the immunotoxicity of chemicals are generally not adequately represented in current routine toxicity testing schemes, and, as a result, only a limited number of chemicals have been shown to have immunosuppressive properties in such tests. Vos (1977) suggested that these chemicals may be representative of a much larger group of immunotoxic chemicals and advocated the inclusion of techniques for the evaluation of the function and morphology of the immune system in routine test protocols. In order to accomplish this, it is necessary to incorporate knowledge from the expansive, growing immunology discipline into the field of toxicology.

The development of tests to evaluate the immune response during toxicological testing is gaining interest. Older in vivo test methods, which can be expensive and cumbersome, are undergoing reevaluation, and in vitro correlates that are cheaper, easier to handle, and more reproducible are being developed.

The primary purpose of this section is to review those tests of immune function, immunocompetence in particular, that appear to be suitable for inclusion in routine toxicity testing batteries. In addition to tests for immunocompetence, tests for the induction of clinical allergy (those diseases in which hypersensitivity plays a part, such as contact hypersensitivity, hay fever, and asthma) will be mentioned briefly. Recommendations for tier testing schemes for immunotoxicity will be presented.

An in-depth review of the organ systems and the complex cellular and humoral interactions that are necessary for, and ultimately result in, an immune response is not within the scope of this document. A glossary of immunological terms, prepared for the nonimmunologist, is provided at the end of the summary. For a more comprehensive survey of basic immunology, the references listed in Section 3.8 are recommended.

3.2 TESTS TO DETECT ALTERATIONS IN THE IMMUNE RESPONSE

Chemicals capable of inducing alterations in the immune response may alter both specific and nonspecific responses, and the response may be enhanced or suppressed, depending on a variety of circumstances that include chemical and dosage level and the species and age of the test animal (Luster and Faith 1979). For example, inorganic metals have been shown to affect primarily humoral immune responses (Koller 1979a) while halogenated aromatic hydrocarbons affect cell-mediated immunity; low-level exposure to certain chemicals may lead to immunoenhancement while higher levels are immunosuppressive (Koller et al. 1977, Holt and Keast 1977); hexachlorobenzene is reported to produce opposite responses in the rat and the mouse (Moore 1979); and the developing immune system may be more sensitive to the effects of environmental chemicals than the adult immune system (Vos and Moore 1974, Moore and Faith 1976).

It has generally been thought that no agent selectively suppresses the antibody-forming mechanism but that any agent that suppresses cell proliferation or interferes with protein or nucleic acid formation will, under proper circumstances, suppress the antigen-antibody mechanism (Loomis 1974). However, there is evidence to suggest that this is not necessarily the case, but that the various cell types that participate in the immune response can be specifically depressed (Nicolin et al. 1980).

When a chemical compound is suspected of altering the immune response, both the cellular and humoral systems must be tested (Koller 1979a). Tests of cell-mediated immunity generally measure the specific activities of the T-lymphocyte populations and may be performed using in vivo or in vitro techniques. Tests of humoral immunity generally measure B-cellular synthesis of antibody and circulating antibody, primarily B-lymphocyte properties. There are also tests to measure macrophage properties and other indirect parameters of the immune system. Thus, tests are available to help determine which of the following steps in the immune response are altered by a particular chemical (Vos 1977):

1. antigen processing (phagocytosis),
2. antigen recognition by lymphocytes,
3. adaptive phase (differentiation and proliferation of T- and B-lymphocytes), or
4. effector phase.

Representative tests, primarily the original techniques or the modifications of those techniques that seem suitable for toxicity screening, are listed in Table 3.1 and are reviewed in the following sections.

As in any discussion of toxicity testing procedures, the importance of proper test conditions should be recognized. Factors that may affect the outcome of the tests are listed in Table 3.2 and are discussed in detail by Doull (1975).

3.2.1 Assessment of Cell-Mediated Immunity

The main categories of cell-mediated immunity include: classical cell-mediated protective immunity, which is mainly effective against protozoal, viral, fungal, and some bacterial infections; delayed hypersensitivity skin reactions to extracts or whole suspensions of organisms, also identified as tuberculin hypersensitivity; chemical contact sensitivity; allograft rejection; immunological surveillance to tumors; and certain organ-specific autoallergic diseases, such as thyroiditis, encephalomyelitis (following rabies vaccination), adrenalitis, and orchitis (Turk 1975, Faith et al. 1980).

T-cells are required for the induction of the cell-mediated immune response. When stimulated by antigen, T-cells form a pool of sensitized, antigen-specific lymphocytes which then can function as memory cells (which produce the secondary immune response), effector cells, killer cells, helper cells, or suppressor cells (Faith et al. 1980). T-cells do not produce antibody (Koller 1979a).

Effector cells, in delayed-type hypersensitivity, produce biologically active products called lymphokines. Lymphokines are soluble nonantibody products of lymphocyte activation by antigen and mitogens which are thought to act as molecular mediators of cellular immune responses (Morely et al. 1978). Lymphokines exhibit the following characteristics: (1) produce increased vascular permeability following intradermal injection; (2) increase tritiated thymidine uptake by lymphocytes in culture; and (3) inhibit macrophage migration in vitro. Lymphokines retain biological activity after removal of the antigen that stimulated them. They may also be involved in both expression and regulation of lymphoid cell activity, and they may represent a pathway for the expression of T-cell function. Some lymphokines, such as the migration inhibition factor, the chemotactic factor, and the macrophage-activating factor, influence macrophage

Table 3.1 Tests for Immunotoxicity

Assessment of cell-mediated immunity

- In vivo tests
  - Tests for delayed hypersensitivity
    - Cutaneous hypersensitivity to tuberculin
    - Radiometric ear test
    - Footpad assay
    - Allograft rejection
  - Graft-vs-host reaction
  - In vitro tests
    - Transformation of lymphocytes by mitogens
    - Mixed lymphocyte culture
    - Macrophage migration inhibition factor (MIF)
    - Macrophage aggregation
    - Leukocyte adherence inhibition (LAI)

Assessment of humoral immunity

- Assays for local production of antibody
  - Plaque-forming assays
  - Immunofluorescence
- Measurement of circulation antibody and immunoglobulins
  - Precipitation
  - Hemagglutination and hemolysis
  - Passive hemagglutination
  - Enzyme-linked immunosorbent assay (ELISA)
- Other tests of B-cell function
  - EAC rosette technique
  - Transformation of lymphocytes by B-cell mitogens

Assessment of indirect parameters of immunity

- Macrophage functions
  - Ingestion
  - Intracellular killing
- Host resistance to infection

Tests to detect the sensitizing potential of chemicals

- Skin sensitization
  - Draize test
  - Freund's complete adjuvant test
  - Guinea pig maximization test
  - Split adjuvant technique
  - Buhler test
  - Open epicutaneous test
- Respiratory sensitization

Table 3.2 A Classification of Toxicity-Influencing Factors

1. **Factors Related to the Toxic Agent**
   - Chemical composition (pH, choice of anion, etc.)
   - Physical characteristics (particle size, method of formulation, etc.)
   - Presence of impurities or contaminants
   - Stability and storage characteristics of toxic agents
   - Solubility of the toxic agent in biologic fluids
   - Choice of the vehicle
   - Presence of excipients: adjuvants, emulsifiers, surfactants, binding agents, coating agents, coloring agents, flavoring agents, preservatives, antioxidants, and other intentional and nonintentional additives
2. **Factors Related to the Exposure Situation**
   - Dose, concentration, and volume of administration
   - Route, rate, and site of administration
   - Duration and frequency of exposure
   - Time of administration (time of day, season of the year, etc.)
3. **Inherent Factors Related to the Subject**
   - Species and strain (taxanomic classification)
   - Genetic status (littermate, siblings, multigeneration effects, etc.)
   - Immunologic status
   - Nutritional status (diet factors, state of hydration, etc.)
   - Hormonal status (pregnancy, etc.)
   - Age, sex, body weight, and maturity
   - Central nervous system status (activity, crowding, handling, presence of other species, etc.)
   - Presence of disease or specific organ pathology
4. **Environmental Factors Related to the Subject**
   - Temperature and humidity
   - Barometric pressure (hyper- and hypobaric effects)
   - Ambient atmospheric composition
   - Light and other forms of radiation
   - Housing and caging effects
   - Social factors
   - Chemical factors

Source: Adapted from Doull (1975).

functions (Faith et al. 1980). The properties of lymphokines or products of activated lymphocytes (PALs) have been summarized by Bloom et al. (1974) in Table 3.3.

Killer cells are cytotoxic and participate in the rejection of tumor cells and transplants.

Helper cells interact with B cells in antibody response against many antigens, while *suppressor T-cells* function to suppress the functions of B-cells or effector T-cells. [There is also evidence for the existence of B-cells that modulate T-cells (Turk 1975).]

The induction and manifestation of the cell-mediated immune response, illustrated in Figure 3.1, proceeds as follows (Park and Good 1974, Miller 1975, Luster et al. 1982b):

1. Antigen interacts with T-cells, usually outside the lymphoid tissues (at the site of antigen accumulation or within a graft).
2. Lymph nodes draining the site of sensitization become enlarged, and on day two of the response the small T-lymphocytes in the thymus-dependent areas differentiate to large pyroninophilic cells and divide to produce a progeny of effector cells (small lymphocytes), which enter the circulation after 3 to 4 days. The onset of delayed hypersensitivity coincides roughly with the appearance of these cells in the circulation.
3. The small sensitized lymphocytes (effector cells) now in the circulation come in contact with the antigen and are stimulated to produce lymphokines or effector molecules, which recruit nonspecific cells, such as monocytes, to the site of the reaction.
4. Those monocytes that accumulate are then transformed to macrophages, which through the release of lysozymal enzymes produce various effects leading to the ultimate expression of the cellular response—a delayed hypersensitivity lesion, a graft rejection, or a microbiocidal effect.

In general, in vivo assays of cell-mediated immunity mainly measure the effector mechanism, while in vitro assays may measure cellular components (Luster et al. 1982a). In vivo and in vitro assays that are commonly employed to measure T-lymphocyte functions will be described. These will be discussed with respect to preferred test species, advantages and disadvantages, usefulness in detecting the effects of environmental chemicals, and, when possible, applicability of the test to particular chemical classes.

3.2.1.1 In vivo tests

Basic in vivo procedures for evaluating the cell-mediated immune response include tests of delayed hypersensitivity, allograft rejection, and graft-vs-host reactions. These are well-established and valuable procedures,

Table 3.3 Products of Activated Lymphocytes (PALs) In Vitro

A. **Affecting Macrophages**	
Migration Inhibitory Factor (MIF)	Inhibits migration of normal macrophages
Macrophage Aggregation Factor	Agglutinates macrophages in suspension
Macrophage Spreading Factor	Increases adherence and surface area of macrophages
Macrophage Activating Factor (MAF)	Increases glucose-C-1 oxidation and phagocytosis
Macrophage Chemotactic Factor	Causes macrophages to migrate along gradient through micropore filter
Cytophilic Antibodies	Confer some specific reactivity to antigens
B. **Affecting Lymphocytes**	
Blastogenic or Mitogenic Factors (BF, MF)	Induce blast cell transformation and thymidine uptake by normal lymphocytes
Thymus Replacing Factor (TRF) or Helper Factor (HF)	Permits differentiation of B cells to Ab secreting cells
Suppressor Factor	Inhibits activation of, or antibody production by, B cells
Transfer Factor (TF)	Converts nonreactive cells to cells capable of reacting to specific antigens
C. **Affecting Neutrophils**	
Leucocyte Inhibitory Factor (LIF)	Inhibits migration of polymorphs
Leucocyte Chemotactic Factor	Causes polymorphs to migrate through micropore filter
D. **Affecting Eosinophils**	
Eosinophil Chemotactic Factor (ECF)	Together with Ab-Ag complex, causes migration through micropore filter
Eosinophil Promoting Factor	Increases migration from agarose drops
E. **Affecting Other Cell Types**	
Lymphotoxin (LT)	Cytotoxic, especially for L-cells
Proliferation Inhibitory Factor (PIF)	Inhibits proliferation without killing cells
Cloning Inhibitory Factor (CIIF)	
Osteoclast Activating Factor (OAF)	Causes release of ^{45}Ca from embryonic bone cultures
Colony Stimulating Factor (CSF)	Stimulates differentiation of bone marrow stem cells into granulocytic and/or monocytic cells
Interferon (IF)	Renders cells resistant to virus infection

Source: Adapted from Bloom et al. 1974.

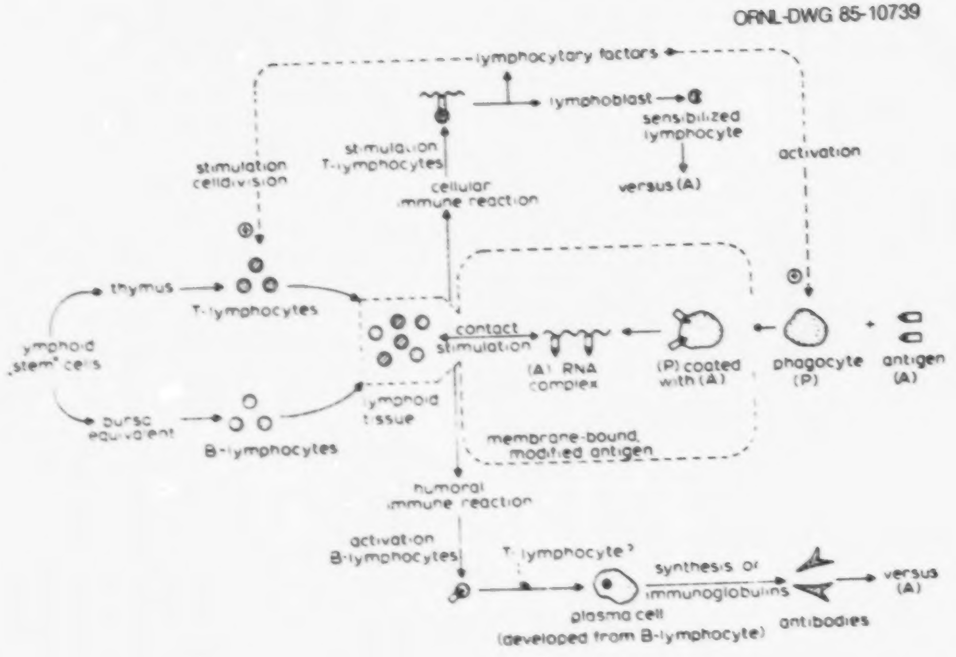
ORNL-DWG 85-10739
lymphocytary factors
lymphoblast
sensibilized lymphocyte
versus (A)
stimulation T-lymphocytes
stimulation celldivision
activation
cellular immune reaction
lymphoid "stem" cells
thymus
T-lymphocytes
bursa equivalent
B-lymphocytes
lymphoid tissue
contact stimulation
(A) RNA complex
(P) coated with (A)
phagocyte (P)
antigen (A)
membrane-bound, modified antigen
humoral immune reaction
activation B-lymphocytes
T-lymphocyte?
synthesis of immunoglobulins
plasma cell (developed from B-lymphocyte)
antibodies
versus (A)

Figure 3.1. Hypothetical mechanisms for cellular and humoral immune reactions. (Source: De Bruin 1976. Used with permission of the publisher.)

but they are generally cumbersome and time consuming and require large numbers of animals. Newer tests have been introduced, some utilizing isotopes, that are quantitative and more sensitive. Some of these have been recommended for inclusion in toxicity screening protocols.

Tests of delayed-type hypersensitivity. Cell-mediated immunity is often measured by delayed hypersensitivity reactions (Koller 1979a). The evaluation of delayed hypersensitivity is generally based on dermal reactions that lead to erythema, induration, and increase in skin thickness. More recent tests have been introduced that are based on the radioactive labeling of bone marrow precursors of monocytes that subsequently migrate into sites of inflammation (Lefford 1974) or on the extravasation of human serum albumin into the area of hypersensitivity response (Paranjpe and Boone 1972). All of these tests are important to the assessment of immunocompetence. Examples are described in the following paragraphs.

Skin Reactions (Useful in Immunotoxicological Studies)

An important test for measuring cell-mediated immunity is the delayed cutaneous hypersensitivity reaction to tuberculin as described by Flax and Waksman (1962). In the original experiment rats were injected in one hind footpad with a suspension of heat-killed tubercle bacilli and then were challenged with a purified protein derivative of tuberculin (PDD), which was injected intradermally into the flank. The animals were observed routinely at 4 to 6, 24, 48, and 72 h, and the diameter and degree of induration of the skin reactions were determined. The reaction was maximal at 24 to 48 h.

In addition to the rat, the guinea pig is often used in this system, and in the past was considered to be the species of choice.

The usefulness of this skin test in the evaluation of immunotoxicologic properties of chemicals, as well as species specificity of the test, was demonstrated by Vos et al. (1973) and by Vos et al. (1979b). The procedure used was similar to that of Flax and Waksman (1962); for a higher degree of quantitation, the thickness of the reaction was measured as an end point, in addition to diameter and degree of induration. In the study by Vos et al. (1973) the skin test reaction was evaluated in guinea pigs and rats following administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Guinea pigs were dosed for 8 weeks with 0.008, 0.04, 0.2, or 1.0 μg/kg (body weight). Skin reactions were significantly reduced at the 0.2- and 0.04-μg/kg levels. All guinea pigs treated with 1.0 μg/kg died or displayed overt signs of toxicity. Thymus atrophy and lymphopenia were observed. The rats were dosed for 6 weeks with higher doses of 0, 0.2, 1.0, and 5.0 μg/kg, and, although animals at the 5-μg/kg level had lower body, thymus, and adrenal weights, the skin reactions were not altered.

In the second study, Vos et al. (1979b) included the skin test in a battery of immunological assays to test the effects of hexachlorobenzene in the rat. The cell-mediated immune response was not altered by the chemical, but the antibody response was stimulated.

The cutaneous hypersensitivity reaction to tuberculin is not a very sensitive test for measuring cell-mediated immunity (Vos 1977), and measurements of the reaction are extremely subjective and vary considerably between observers (Carter and Bazin 1980). However, the skin test offers several test sites per animal, is quick and easy to perform (Crowle 1975), and can yield reproducible results with little variation if careful measurements are made. Lefford (1974) introduced a new test using radiolabeling techniques, which is more quantitative and less subjective than the more traditional skin test. This procedure is described in the following section.

Radiometric Ear Test

The Lefford (1974) technique for measuring delayed-type hypersensitivity (DTH) is based on the radioactive labeling of bone marrow precursors of monocytes, which subsequently migrate to sites of inflammation and accumulate in DTH reactions. Rats were sensitized intravenously or subcutaneously with viable bacillus Calmette-Guerin (BCG), then injected subcutaneously with thymidine methyl-^{3}H. Twenty-four hours later the animals were injected in the left ear with tuberculin purified protein derivative (PPD). The right ear, injected with diluent, served as a control. Twenty-four hours after the injection of antigen the central portion of each ear was removed, and radioactivity of the tissue was measured in a liquid scintillation spectrometer. Counts of the test ear were then compared with those of the control ear, and it was possible to calculate the left ear/right ear ratio as the parameter of delayed hypersensitivity.

Mice and guinea pigs may also be tested by this method, and various protein antigens, other than PPD, can be used; however, PPD is recommended for rats and guinea pigs while other protein antigens, including bovine serum albumin, are generally used in mice (Luster and Faith 1979).

Moore and Faith (1976) introduced a modification of the Lefford method in which rats were sensitized to oxozalone by skin-painting the ears. Tritiated thymidine was injected intraperitoneally 10 days later, and 24 h after thymidine injection a challenging dose of oxozalone in olive oil was painted on one ear. Standard procedures were then used to determine the ratio of radioactivity in the treated and untreated ears. Moore and Faith (1976), by using this method, were able to detect suppression of cell-mediated immunity in Fischer rat pups exposed to TCDD on gestation day 18 and on postnatal days 0, 7, and 14.

In a later study, Fraker (1980) used a similar technique to test the effects of polybrominated biphenyls (PBB) in mice. The animals (fed PBB) were sensitized percutaneously and challenged on one ear lobe with dinitrofluorobenzene. After intravenous injection of ^{125}I-deoxyuridine, the ratio of radioisotope incorporated in the challenged versus the unchallenged ear lobe served as a measure of T-cell-dependent delayed hypersensitivity. No effect was seen on this response. This was of particular interest because, in the same study, the IgG and IgM plaque-forming cells were impaired by PBB, indicating that both helper T- and B-cells were affected. T-cells involved in delayed-type hypersensitiviy and those involved in humoral immunity are of the same phenotype and probably follow the same developmental pathway; thus the differential effects of PBB were thought to be due to differences in susceptibility to the chemical of the various subclasses of T-cells (Fraker 1980).

There are certain disadvantages to this method: first, each ear can be tested only once, so each animal can receive a maximum of two tests; furthermore, if the left ear/right ear ratio is calculated the animal can be used only once. Advantages of the technique are objectivity, reproducibility, and sensitivity, and these far outweigh the disadvantages. It appears to be particularly useful as a general screen for chemical effects because the participation of many cell types is required for a normal reaction.

Footpad Assay

The footpad assay is based on the observation that ^{125}I-human serum albumin (^{125}I-HSA) will extravasate into the edematous area produced by a delayed hypersensitivity response (Paranjpe and Boone 1972). In the procedure described by Kauffmann et al. (1982), Sanders et al. (1982), and Munson et al. (1982), recommended for testing the effects of chemicals on cell-mediated immunity, mice are immunized in the left hind footpad with sheep red blood cells (SRBC). Four days later the mice are challenged in the same footpad with SRBC and 17 hours later are injected intravenously with ^{125}I-HSA. The animals are killed and both feet are removed and measured for radioactivity in a gamma scintillation counter. Control animals are not sensitized but are challenged in order to determine nonspecific swelling. The stimulation index (S.I.) is calculated:

$$\text{S.I.} = \frac{\text{Left footpad -}}{\text{Right footpad}} \; \bar{x} \; \frac{\text{Left footpad unsensitized}}{\text{Right footpad unsensitized}}$$

Minor aspects of the technique, such as the number and route of immunizing injections, the timing of the challenge, and timing of the isotope injection, may vary between laboratories. Also, footpad swelling instead of extravasation of isotope may be the end point of the assay (Muller et al. 1977). Mice are generally used in this assay. However, it has been noted that the strain of mouse may influence detectability of the response (Crowle 1975).

Muller et al. (1977) used the footpad assay to demonstrate the suppressive effects of lead on DTH in mice. Alterations of both primary and secondary responses to SRBC were correlated with the concentrations of lead in the blood. In another study, DTH, assayed by footpad injection, was also shown to be suppressed in male mice by trichloromethane and in males and females by bromodichloromethane (but not by bromoform and dibromochloromethane) (Schuller et al. 1978). Smith et al. (1978) reported a similar inhibition of the footpad response with polychlorinated biphenyls (Aroclor 1254). Sanders et al. (1982), using the footpad assay, observed a suppression of the stimulation index by subchronic administration of trichloroethylene in female (but not male) mice.

According to Dean et al. (1979a), the footpad assay is probably the best single parameter for immunologic competence because it measures both the recognition and effector phases of the cell-mediated immune response and requires the cooperation of the macrophage/monocyte series.

The isotope system is sensitive and quantitative, and the reaction appears to be of the pure delayed type without the humoral component of the immediate anaphylactic or arthus type, which often accompanies the delayed hypersensitivity reaction (Paranjpe and Boone 1972).

The assay can be used to determine the effects of chemicals on the ability of the host to be immunized or to respond to challenge after immunization (Dean et al. 1979a).

Allograft Rejection. Skin grafting is a useful technique that has been applied to the study of various biological problems. The assay is dependent upon the ability of an experimental host animal to reject a tissue transplanted from a donor that differs from the host at an important histocompatibility locus. Allograft rejection time is used as the end point for detecting alterations in the cell-mediated immune response of experimental animals.

Standard techniques for skin grafting in rabbits, guinea pigs, and mice were described by Billingham and Medawar (1951). These classic methods can be laborious and time consuming and are not practical for situations that require large numbers of grafts.

A rapid method of grafting skin on tails of mice was described by Baily and Usama (1962). This widely used procedure consists of slicing an approximately 5- by 2-mm section of skin from the tail and transferring it to a raw area of the host tail from which the reciprocal graft has just been removed. Grafts are placed on the host so that the direction of hair growth is opposite to that of the host in order that "takes" can be distinguished from possible host replacement. The grafts adhere well and do not require extraneous pressure or dressing. Also, they are protected by, and can be observed through, a length of glass tubing fitted over the tail.

This technique can also be successfully performed on rats. Using allograft rejection as one of the parameters, Vos and Moore (1974) detected impairment of cell-mediated immunity in the offspring of rats treated, during gestation and postnatally, with TCDD. In a more recent study, Vos et al. (1979b) incorporated allograft rejection into a battery of function tests used to ascertain the effects of hexachlorobenzene on the immune system of the rat. The chemical had no effect on the in vivo cell-mediated immune response but did enhance humoral immunity.

The tail skin-grafting technique is simple and rapid, and a particular advantage is that the graft is in plain view at all times; however, the assay is not applicable to studies requiring the use of large grafts or host areas other than skin, and it is not particularly quantitative.

Graft-vs-host reaction. In the graft-vs-host reaction (a classic assay of cell-mediated immunity), immunocompetent cells are injected into hybrids of parental strains of animals that differ from each other at the major histocompatibility locus. The donor cells do not possess any antigens foreign to the host and thus are tolerated by the hybrids. However, the immunocompetent donor cells do react against the foreign antigen in the host and, therefore, induce a host reaction that can be measured by several methods, including the spleen weight assay and the renal enlargement assay. These methods have been reviewed by Ford (1978). In immunotoxicology studies the test compound is given to the donor animals.

A particularly useful technique was described by Ford et al. (1970) in which donor spleen or thoracic duct lymphocytes were injected into the feet of young F_1 hybrid rats. The recipients were killed seven days later, and the weights of their popliteal lymph nodes were plotted against the dose of cells injected, the degree of enlargement of the draining lymph nodes being a measure of the graft-vs-host activity of the donor cells.

Differences in the effects of various chemicals on the immune response have been demonstrated with the graft-vs-host popliteal lymph node assay. For example, TCDD suppressed the response in rats and mice (Vos et al. 1973, Vos and Moore 1974), while Aroclor 1016 and hexachlorobenzene enhanced the response.

The popliteal lymph node assay is a sensitive and quantitative measure of cell-mediated immunity. Its in vitro analog, the mixed lymphocyte culture reaction, is discussed in a later section.

3.2.1.2 In vitro tests

In vitro tests of cell-mediated immunity were initially developed for the purpose of analyzing the mechanisms of the response (Bloom et al. 1974), but it soon became evident that these methods for testing cell-mediated immunity also offered possibilities, at the clinical level, to objectively assess the status or level of delayed hypersensitivity without risk to the patient. These assays are also useful in the detection of alterations in the immune response of experimental animals following exposure to potential immunotoxicants. Those tests that seem most suitable for this purpose are described.

Transformation of lymphocytes by mitogens. The ability of cultured lymphocytes to undergo transformation and to incorporate labeled thymidine (^{3}H-TdR) into DNA following nonspecific stimulation with mitogens is the basis of one of the most valuable tools of immunotoxicology. The mitogenic response of lymphocytes is a parameter of the adaptive phase of the immune response (Vos et al. 1979c). Some mitogens selectively stimulate the cellular immune (T-cell) components while others stimulate the humoral immune (B-cell) components. Phytohemagglutinin (PHA) and concanavalin A (Con A) are the T-cell mitogens of choice (Luster and

Faith 1979), while lipopolysaccharides seem to stimulate primarily B-cells (Smith 1972). On the other hand, pokeweed mitogen stimulates both B- and T-cells and can be used to assay peripheral blood lymphocytes that do not respond to other mitogens (Luster and Faith 1979). The response of lymphoid cells to Con A and PHA is discussed in greater detail by Stobo et al. (1972).

Thorpe and Knight (1974) describe a microplate culture technique for lymphocyte transformation and define optimal conditions for performing the assay. Essentially, various concentrations of mouse mesenteric lymph node cells were cultured in microplate wells in the presence of mitogens. The plates were incubated at 37°C in 5% CO_2. Twenty-four hours prior to harvesting, ^{3}H-TdR was added to each culture. Cultures were usually harvested after 72 h and then processed for scintillation counting to measure ^{3}H-TdR incorporation. An alternative test that measures mitogen- (and alloantigen-) induced lymphocyte proliferation in a one-way mixed leukocyte culture was described by Dean et al. (1979a), who advocate its use for defining changes in host immunocompetence.

Examples of the use of mitogen stimulation in immunotoxicology studies are numerous. Mitogen stimulation is usually performed on mouse lymphocytes, because Fischer rats, which are commonly used in toxicology studies, respond poorly to the usual mitogens (Tada et al. 1974, as reported in Luster and Faith 1979). The response to T-cell mitogens correlates well with cell-mediated immunity and can be applied to cells that have been exposed to chemicals in vivo or in vitro. Vos and Moore (1974) tested the mitogen response of lymphoid cells from mice and rats that had been exposed to TCDD at different ages. In rats whose mothers were exposed during gestation and postnatally, the PHA response was reduced on a cell-for-cell basis, but both the PHA and Con A responses recoverable from the whole thymus were depressed. In mice treated with TCDD at 1 month of age, reduced responsiveness of spleen cells to PHA was seen only at a clearly toxic dose level; in 4-month-old mice PHA responsiveness was not depressed. The thymus was the main organ affected by TCDD, and other parameters of cell-mediated immunity were depressed in the very young animals. The data suggest that TCDD-suppression of cell-mediated immunity may be age related. In a later study, Sharma et al. (1978) similarly reported that PHA and pokeweed mitogen responses in mice and rabbits were suppressed with toxic levels of TCDD and, furthermore, that low levels of the chemical caused enhancement of immune reponsiveness similar to that produced by antigenic substances.

A related chemical, 2,3,7,8-tetrachlorodibenzofuran (TCDF), also produced a moderate suppression of the lymphoproliferative response (to PHA and lipopolysaccharides, but not to Con A) of splenic lymphocytes taken from guinea pigs treated orally with the chemical (Luster et al. 1979).

Other industrial chemicals that have been shown to alter the mitogenic response of lymphocytes include polybrominated biphenyl (Firemaster FF-1), which decreased the responsiveness of spleen cells from rats and mice (Moore et al. 1978); dioxane, which suppressed mouse T-cell responses while augmenting B-cell responses (Thurman et al. 1978); and toluene di-isocyanate, which stimulated the response of both human and mouse lymphocytes (Thurman et al. 1978).

Chronic exposure of mice to fresh cigarette smoke resulted in a marked depression of the PHA response of lymphocytes taken from the regional lymph nodes of the respiratory system and from the blood (Thomas et al. 1974a).

The sensitivity of the mitogen response to the effects of heavy metals has been tested by several investigators. In one study, Gaworski and Sharma (1978) observed depressed responses to PHA and pokeweed mitogen in splenic lymphocytes taken from mice fed high, but subtoxic, levels of lead, cadmium, or mercury. Similar effects were observed in rat lymphocyte responses to PHA and Con A following chronic prenatal and postnatal exposure to low levels of lead (Faith et al. 1979). However, in a third study (Koller et al. 1979), lead and cadmium did not significantly affect the response of mouse lymphocyte proliferation by Con A. Koller et al. (1979) suggested that the conflicting results of the two mouse studies could be due to variation in amount and length of exposure to the compounds, strain differences, or differences in the response of lymphocyte subpopulations to PHA and Con A.

Lymphocyte transformation is a semiquantitative and reproducible technique, and the microplate method is practical and labor saving (Vos 1977; Luster and Faith 1979). The procedure has been adapted for use with human cells in clinical laboratories to determine the immune status of patients (Thurman et al. 1978). Luster and Faith (1979) point out, however, that it does not measure effector function nor does it represent a normal response.

Mixed Lymphocyte Culture. The mixed lymphocyte culture (MLC) reaction is an in vitro analog of the allograft rejection response (Thurman et al. 1978, Luster and Faith 1979). Similar to the mitogen stimulation assay, lymphocytes in culture are normally stimulated by allogeneic lymphocytes to undergo blastogenesis, and lymphoproliferation is then measured by the amount of tritiated thymidine incorporated by the test cells. The response, probably a measure of both B- and T-cell proliferation, is stimulated by cell surface antigens and requires macrophages for the response to be initiated (Thurman et al. 1978).

The MLC assay has not been used extensively in immunotoxicology studies. However, the system was employed by Thurman et al. (1978) as one of several parameters examined in the evaluation of the immunotoxicologic effects of toluene diisocyanate, dioxane, and vinyl chloride in mice.

Their procedure was as follows: spleens were removed from untreated CBA/J and C57Bl/6J mice and cell suspensions were prepared; blastogenesis of these cells was blocked with mitomycin. For both control and test groups, responding (unblocked) thymus, spleen, or lymph node cell suspensions were prepared from CBA/J mice that had been treated with the different chemicals, and those cells were cultured in microtiter plates for 72 or 96 h. Control cultures received 0.1 mL of syngeneic, blocked CBA/J lymphocytes, and the test cultures received allogeneic, blocked C57Bl/6J lymphocytes. The cultures were pulse-labeled and prepared for scintillation counting to measure lymphoproliferation of the unblocked CBA/J cells.

The proliferation response of the spleen, thymus, and lymphoid cells was generally inhibited by concentrations of 0.001 to 0.05% toluene diisocyanate but apparently was stimulated by 0.1% toluene diisocyanate. The response was lowered slightly by 10% dioxane and was not affected by vinyl chloride (500 ppm for 7 h).

The assay is fairly simple to perform; spleen, thymus, and lymph node cells can be tested; and treatment with chemicals can be in vitro as well as in vivo, which qualifies the test for use with human cells. However, Oehler et al. (1977) found that it is sometimes difficult to demonstrate an MLC response in spleens from rats because of the presence of suppressor macrophages. Therefore, it has been suggested that blood lymphocytes from most species can be substituted for spleen lymphocytes, following Hypaque-Ficol density gradient centrifugation (Luster and Faith 1979).

Macrophage Migration Inhibition Factor (MIF). Bloom and Bennett (1966) observed that sensitized peritoneal lymphocytes, upon interaction with specific antigen in vitro, elaborated into the medium a soluble material that could inhibit migration of normal exudate cells. In their experiment, peritoneal macrophages, from guinea pigs sensitized to tuberculin with Freund's adjuvant, were drawn into capillary tubes and centrifuged. The capillary tubes were cut at the cell-medium interface and were placed in special chambers containing various supernatants from sensitized peritoneal lymphocyte cultures. Macrophages, exposed to the supernatants from cultures of lymphocytes growing in medium alone, migrated out of the tube onto a specially placed coverslip. Macrophages that were exposed to the supernatant from cultures of lymphocytes growing in purified protein derivative (PPD) did not migrate. The soluble material elaborated into the supernatant by the PPD-stimulated lymphocytes has been identified as the lymphokine MIF. This type of cellular interaction can be detected in experiments typified by the following (David and David 1971):

1. In the simplest migration assay, sensitized peritoneal exudate cells (macrophages and lymphocytes) are assayed in a capillary tube against the appropriate antigen.

2. In another assay, lymphocytes from lymph nodes or other organs are mixed with normal peritoneal exudate cells in order to detect sensitive cells in the various organs.

3. In the "indirect" assay, lymphocytes are incubated with antigen, and the cell-free supernatants are assayed for MIF activity using normal peritoneal exudate cells.

The in vitro migration system has been used primarily in guinea pigs, but also in mice, rats, hamsters, and monkeys (David and David 1971); and it has been adapted to the assay of human cells (Thor 1971).

The principle of this reaction has been applied to the measurement of the effector stage of cell-mediated immunity in immunotoxicological studies (Luster and Faith 1979). For example, Luster et al. (1979) used an in vitro MIF assay to demonstrate a modest, but dose-related suppression of cell-mediated immunity in TCDF-exposed lymphocytes. These results were in agreement with the mitogen stimulation assay, another in vitro test of cell-mediated immunity.

The migration assay is semiquantitative and not extremely expensive; however, it does require attention to technical detail (Luster and Faith 1979, Morley et al. 1978). Assay (1), above, is thought to be more sensitive than other in vitro methods of assessing delayed hypersensitivity provided high concentrations of antigens are used (Carter and Bazin 1980).

Macrophage Aggregation. Macrophage aggregation, another in vitro correlate of DTH, may become more widely used than MIF tests because of its relative simplicity (Crowle 1975).

During investigations of the interaction of antigen and sensitive lymphoid populations, aggregation of sensitive peritoneal exudate cells was observed in tubes in the presence of antigen. Lolekha et al. (1970) studied this reaction using BCG and egg albumin as antigens. Guinea pigs were immunized via footpad injections, and the test was performed 4 to 8 weeks following BCG immunization and 10 to 14 days after egg albumin. The animals were injected intraperitoneally with light mineral oil and 5 days later were exsanguinated. Peritoneal exudate cells were removed by conventional methods and were washed, counted, and resuspended in sterile tubes containing PPD or egg albumin. After 24 h at 37°C and 5% CO_2 the tubes were shaken to resuspend the cells for observation of aggregates. The reaction was antigen-specific, and it was also found that supernatant fluids from lymphoid cells cultured from antigen-sensitive animals caused aggregation of nonsensitive peritoneal exudate cells in the presence of antigen. The investigators concluded that exposure to antigen leads to the release of a macrophage aggregation factor (MAF) from sensitive lymphoid cells in culture.

The soluble material MAF, which causes aggregation of peritoneal exudate cells may be the same as MIF (Lolekha et al. 1970). The MAF assay is simpler and less time consuming than the MIF assay (Lolekha et al. 1970, Crowle 1975) and lends itself to semiquantitation by routine titration.

No examples were found for the use of this test in immunotoxicology studies.

Leukocyte Adherence Inhibition (LAI). This assay, originally described by Halliday and Miller (1972), measures the release of a diffusable factor from sensitized T-lymphocytes following contact with antigen. The factor, which inhibits the adherence of leukocytes to glass or plastic surfaces, has been used to assess cell-mediated immunity. A modified microtest, adapted from Halliday and Miller by Holt et al. (1974), was used in the following study to detect immunological effects of cigarette smoke.

In this study (Holt et al. 1976) mice were exposed to high- or low-tar cigarette smoke for 36 or 31 weeks, respectively, then inoculated with BCG. Purified protein derivative of BCG was dispensed into microtest tissue culture plates, and splenic lymphocytes from the BCG-treated mice were added to the plates. After a 2-h incubation period the plates were washed and stained, and adherent cells were counted. The numbers of adherent cells in the wells containing antigen were compared with the numbers in wells containing no antigen. Exposure to high- and low-tar cigarette smoke resulted in suppression of the LAI, indicating suppression of cell-mediated immunity.

The reaction of LAI is similar to that of macrophage migration inhibition, but the procedure is much simpler to perform (Halliday and Miller 1972).

3.2.2 Assessment of Humoral Immunity

Humoral immunity is defined as that specific immunity which is mediated by antibodies (immunoglobulins) present in the plasma, lymph, and tissue fluids of the body and which may also become attached to cells (Herbert and Wilkinson 1971). The cells ultimately responsible for the secretion of antibody are the B- (bursa-derived; bone marrow-derived) lymphocytes. When stimulated by antigen, B-cells, specific for that antigen, either proliferate and develop into plasma cells (antibody-secreting cells) or serve as memory cells.

The introduction of antigen is usually followed by a lag phase when no antibody can be detected in the circulation (Park and Good 1974). During this period (the first 24 to 48 h after antigen) the following occur: antigen-processing by macrophages, active synthesis of mRNA and rRNA, transformation of the lymphocytes, and proliferation of these cells, which differentiate into antibody producers. Morphologic changes occur in the red pulp or at the edges of the white pulp of the spleen or in the medullary cords or zones surrounding the germinal centers in the cortical areas of the lymph nodes.

The lag phase is followed by a logarithmic phase during which the rate of production of 19S IgM antibody, the first detected, follows a logarithmic curve. After IgM production, 7S IgG antibody appears and enters the log phase. In a typical antibody response the plateau of antibody production is reached about 8 days after introduction of antigen, and the decay period which then follows is short. The primary response is weak, sluggish, and short lived.

In contrast to the primary response, the secondary response (antibody production after the second immunization) is characterized by faster development and higher levels of antibody titers and a predominance of IgG early in the response.

It has been determined (Robertson 1981) that immunoglobulin M is synthesized by the B-cells at early stages of maturation and that IgG, IgA, or IgE are synthesized at later stages.

For most antigens, cooperation between both T- and B-cells is necessary for the induction of antibody-formation (this is the T-cell-dependent humoral response) (Claman et al. 1966, Miller and Mitchell 1968). However, certain antigens, such as type III pneumococcal polysaccharide and *E. coli* lipopolysaccharide, can stimulate a good immune response in athymic animals (e.g., nude mice) (this is the T-cell-independent humoral response) (Moller and Michael 1971, Andersson and Blomgren 1971). Tests of humoral immunity include those which measure circulating antibody or local production of antibody to both thymus-dependent and thymus-independent antigen; those which identify and quantitate the various immunoglobulins; and those which assess the colony-forming and blastogenic capabilities of B-cells. Although numerous useful tests (and modifications of these tests) have been developed for the purpose of evaluating the humoral immune response, the following discussion will be limited to those which, because of convenience, speed, sensitivity, etc., appear to be likely candidates for inclusion in toxicity-screening protocols.

3.2.2.1 Assays for local production of antibody

Many methods have been developed for the enumeration of individual cells producing antibody. Dresser (1978) defined three approaches to the study of these cells with respect to localization of antibody at various sites: (1) detection of antibody as it is secreted from the cell; (2) detection of intracellular antibody; and (3) detection of antibody on the cell surface. Tests designed to identify antibody at these sites include plaque-forming assays (localized hemolysis in gel), immunofluorescence techniques, and the "erythrocyte-antibody-complement" (EAC) rosette assay, respectively.

Plaque-forming assays (localized hemolysis in gel). Jerne and Nordin (1963) first described this method for enumerating antibody-producing cells, and it has become one of the most widely used tests in experimental immunology.

According to the original assay, cells are obtained from the spleen or other lymphoid organs of an animal that has been immunized against SRBC. These cells are mixed with SRBC and molten agar, the mixture is poured into a petri dish and allowed to set, and the dish is incubated at 37°C. After incubation the release by each cell of hemolysins (antibodies capable of lysing red blood cells in the presence of complement) is revealed with the addition of complement to the dish. A clear zone of hemolysis appears around each antibody-forming cell.

The technical aspects of the plaque-forming cell assay have been described in detail by Dresser (1978).

The PFC technique of Jerne and Nordin (1963) detected only those cells producing antibody, primarily IgM, which could fix complement and lyse cells directly. These cells are referred to as direct plaque-forming cells. Other classes of immunoglobulins (IgG, IgA) are either secreted in insufficient amounts or are incapable of fixing complement, unless they are complexed with antiglobulin antibody. Cells that produce these antibodies are referred to as indirect plaque-forming cells and are revealed when developing serum is added to the plates. The PFC technique can be used to measure both primary and secondary responses.

Modifications of the original technique have been introduced that are more convenient, sensitive, and economical than the original procedure. Methods have been described that utilize agarose instead of agar as the support medium (Plotz et al. 1968, Yamada and Yamada 1969, Duke and Harshman 1971, Urso and Gengozian 1973) or that use no support medium at all (Cunningham and Szenberg 1968, Kennedy and Axelrad 1971). Some of these procedures employ microscope slides instead of petri dishes (Plotz et al. 1968, Urso and Gengozian 1973). Three of these assays were compared for sensitivity by Peterman and Wust (1975), who also defined optimal conditions for the assay by testing spleen cells from rats immunized with sheep or mouse erythrocytes in combination with human or guinea pig complement. They found that the most sensitive system was one using SRBC, no agar or agarose, and either human or guinea pig complement.

Other modifications of particular importance to immunotoxicology have been introduced by: (1) Mishell and Dutton (1967), who performed the plaque-forming cell assay following in vitro immunization of normal spleen cells; (2) Archer et al. (1978), who used the Mishell-Dutton modification to develop an assay for the in vitro immunosuppresive effects of water soluble and insoluble chemicals; and (3) Tucker et al. (1982), who adapted the Mishell-Dutton assay for testing chemicals that require metabolic activation.

The plaque-forming cell assay can be performed with a variety of antigens such as polysaccharides, synthetic polymers, proteins, and haptens, which can be bound covalently to indicator erythrocytes (Peterman and Wust 1975). Thus, both T-cell dependent and T-cell independent antigens can be tested with this procedure. Protein antigens are particularly recommended when Fischer rats or guinea pigs are used because these species do not respond well to SRBC (Luster and Faith 1979).

The plaque-forming cell assay and its modifications have been used frequently in immunotoxicology studies. It has been shown to be a sensitive indicator of alterations in the immune response. Table 3.4 lists examples of the plaque-forming cell response in experimental animals following exposure to environmental pollutants.

It is noteworthy that certain of these chemicals were reported to produce suppression of the plaque-forming cell response at dose levels which produce no signs of overt clinical illness [i.e., hexachlorobenzene, polychlorinated biphenyl (Loose et al. 1977, 1978, 1979), polybrominated biphenyl (Fraker 1980), cadmium chloride (Koller et al. 1975)]. In addition, the plaque-forming cell response was depressed by nickel chloride at levels falling below the threshold limit value for humans (Graham et al. 1978).

The plaque-forming cell response was suppressed by all the chemicals listed in Table 3.4 except for nickel oxide (Graham et al. 1978) and the platinum ethylene diamines (Berenbaum 1971). The lack of effect of nickel oxide could be attributed to insolubility of the compound; lack of effect of the platinum ethylene diamines could be attributed to their chemical nature because other platinum diamines were highly toxic to mice at the doses given.

The advantages of the plaque-forming cell assay include the following: (1) it is sensitive to the immunosuppressive effects of industrial chemicals; (2) it is versatile and convenient and can be performed with a variety of antigens; (3) it lends itself well to the analysis of multiple samples; and (4) the rate of antibody secretion can be determined mathematically by measuring the direct plaque size (Luster and Faith 1979).

Immunofluorescence. Antigens and antibodies can be made fluorescent by chemically binding them to a fluorescent dye, most often isocyanate or isothiocyanate of fluorescein (Gell and Coombs 1975). This fluorochrome labeling procedure can provide rapid, accurate localization of the site of antigen-antibody interaction when one of the reactants forms part of a cell, tissue, or other biological structure.

Fluorochromes are substances that will absorb radiation (ultraviolet light) and become excited. The excited molecules emit observable radiation which ceases almost immediately after the exciting radiation is withdrawn.

There are several approaches to fluorescent labeling. The basic procedures for these have been described in detail by Johnson and Holborow (1973). These microscopic methods are especially applicable to localization of γ-globulin and specific antibody. γ-Globulin in cells can be demonstrated with a fluorescent conjugate prepared from antiglobulin serum (direct method) while specific antibody can be shown with the "sandwich" (or indirect) technique, where the antibody-containing cells are first treated with the antigen and then free valencies of the antigen are used to combine a fluorescent specific antiserum (Figure 3.2).

The application of immunofluorescence in the detection of cellular antibodies using the "sandwich" technique involves these basic procedures (Johnson and Holborow 1973):

1. The preparation of the substrate material (cells or tissue) for microscopic examination. (Care must be taken to avoid fixatives that destroy reactivity of immunoglobulins as antibodies. Cryostat or liquid nitrogen methods may be used for tissue sections; dried smears may be used for cell suspensions.)

2. The preparation and immunological characterization of the fluorescent reagents.

3. Staining with the fluorochrome dye. (Care must be taken to keep the slides moist and to remove nonspecifically bound reactants.)

Table 3.4 PFC Response in Experimental Animals Following Exposure to Environmental Pollutants

Chemical	Species	PFC Response	Reference
Hexachlorobenzene	Mouse	Suppressed	Loose et al. 1977[a]
Polychlorinated biphenyl	Mouse	Suppressed	Loose et al. 1978[a]
	Mouse	Suppressed	Loose et al. 1979[a]
Polybrominated biphenyl mixture	Mouse, rat	No effect	Moore et al. 1978
	Mouse	Suppressed	Fraker 1980[a]
Trichloroethylene	Mouse	Suppressed	Sanders et al. 1982
Cigarette smoke	Mouse	Suppressed	Thomas et al. 1973
	Mouse	Suppressed	Nulsen et al. 1974
	Mouse	Suppressed	Thomas et al. 1974b
Benzo[a]pyrene	Mouse[b]	Suppressed	Urso and Gengozian 1980
Carbon and sulfur dioxide	Mouse	Suppressed	Zarkower 1972
Sulfur Dioxide	Mouse	Suppressed	Zarkower 1972
Carbon	Mouse	Suppressed	Zarkower 1972
Carbon dust	Mouse	Suppressed	Zarkower and Morges 1972
Cadmium chloride	Mouse	Suppressed	Koller et al. 1975[a]
	Mouse	Suppressed	Graham et al. 1978
Chromium chloride	Mouse	No effect	Graham et al. 1978
Nickel chloride	Mouse	Suppressed	Graham et al. 1975
	Mouse	Suppressed	Graham et al. 1978[c]
Nickel sulfate	Mouse	Suppressed	Graham et al. 1978
Nickel oxide	Mouse	No effect	Graham et al. 1978
Platinum diamine dichloride	Mouse	Suppressed	Berenbaum 1971
Platinum diamine tetrachloride	Mouse	Suppressed	Berenbaum 1971
Platinum ethylene diamines	Mouse	No effect	Berenbaum 1971
Methyl mercury	Mouse	Suppressed	Koller et al. 1977
	Mouse	Suppressed	Ohi et al. 1976
Lead	Rat	Suppressed	Luster et al. 1978
	Mouse	Suppressed	Koller and Kovacic 1974

[a] Some doses produced immunosuppression without signs of overt clinical illness.
[b] Exposed in utero.
[c] Doses below threshold limit value.

4. Microscopy under conditions suitable for observing fluorescence. (To achieve maximal fluorescence, a careful choice of filters is necessary, based on the excitation and emission characteristics of the fluorochrome in question.)

Fluorescent techniques have been used successfully in immunotoxicology studies, and their versatility is illustrated by the following examples.

Verschuuren et al. (1970) used a fluorescent-antibody technique to demonstrate a decrease in immunologically active cells in the popliteal lymph nodes of guinea pigs which were fed triphenyltin acetate (TPTA) for 90 days and then injected with tetanus toxoid. The popliteal lymph

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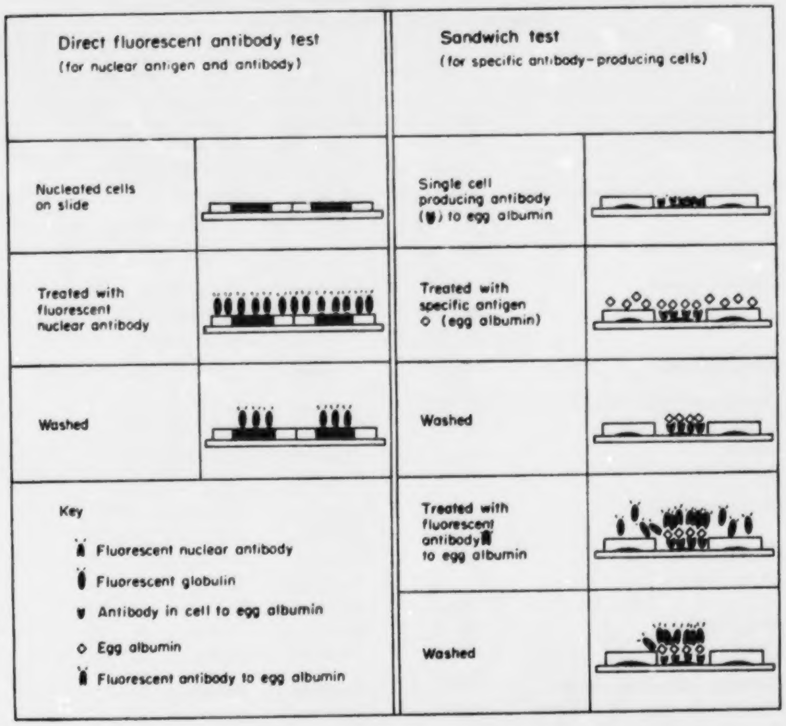
Direct fluorescent antibody test
(for nuclear antigen and antibody)
Sandwich test
(for specific antibody-producing cells)
Nucleated cells on slide
Treated with fluorescent nuclear antibody
Washed
Single cell producing antibody (♥) to egg albumin
Treated with specific antigen ◇ (egg albumin)
Washed
Treated with fluorescent antibody to egg albumin
Washed
Key
Fluorescent nuclear antibody
Fluorescent globulin
Antibody in cell to egg albumin
◇ Egg albumin
Fluorescent antibody to egg albumin

Figure 3.2. Direct fluorescent antibody test and "sandwich test" for staining antibody-producing cells. (Source: Gell and Coombs 1975. Used with permission of Blackwell Scientific Publications, Ltd.)

nodes were subsequently removed and quick-frozen. For detection of all gamma-globulin-containing cells, the cryostat sections were treated with fluoresceinated rabbit anti–guinea pig gamma globulin. For the specific detection of tetanus antitoxin-producing cells, serial sections were treated first with tetanus toxoid, then with rabbit tetanus antitoxin serum, and finally with fluoresceinated goat anti-rabbit serum.

In a later study, Vos and de Roij (1972) used a similar technique to determine the effect of PCB on gamma globulin synthesis in popliteal lymph nodes of guinea pigs stimulated with tetanus toxoid. Photographs were taken of the fluorescein-labeled tissue sections using a fluorescence microscope, and the photographs were ranged in order of increasing number of γ-globulin-containing cells to make statistical evaluation possible. The investigators made the observation that this technique is a sensitive parameter for the detection of immunosuppressive activity of PCB in tetanus toxoid-stimulated animals.

Bozelka et al. (1977) used a direct immunofluorescence method to elucidate the mechanism of cadmium-induced splenomegaly in mice. Following SRBC injection, increased numbers of cells labeled with goat antimouse IgM and IgG were observed in both the white and red pulp of the enlarged spleens of cadmium-intoxicated animals. This led the authors to conclude that the B lymphocyte was responsible for the hyperplastic changes induced by cadmium.

Fluorescent techniques require special equipment, which may be expensive. Also, unwanted fluorescein staining may occur with both the direct and indirect methods. However, the procedure is rapid and specific and can be performed on tissue sections so that antibody-producing cells can be observed in situ. In toxicity testing the procedure would be especially valuable in answering specific questions regarding antibody synthesis once immunotoxicologic effects have been established.

3.2.2.2 Measurement of circulating antibody and immunoglobulins

The production and secretion of antibody is a highly specific response of plasma cells that have been exposed to antigen. Analysis of serum antibody provides a direct and quantitative measurement of the production of specific antibody by these cells. Most procedures require the interaction, in vitro, of antigen and antibody; these procedures can be applied to measurement of the primary and secondary responses to both thymus-dependent and thymus-independent antigens. These procedures, such as routine hemagglutination and hemolysin titrations, are simple to perform and are widely used. More sophisticated methods, such as radial immunodiffusion and the enzyme-linked immunosorbent assay, have been applied to the identification and quantitation of the individual immunoglobulins present in the antisera.

Several of these procedures, sensitive to alterations in humoral immune functions, will be described in the following sections.

Precipitation. When soluble antigen is mixed with antiserum specific for that antigen a precipitate is formed in a few minutes that can be observed and measured (Gell and Coombs 1975). The *interfacial* or *ring technique*, a qualitative test for precipitating antibodies, was developed by Ascoli in 1902 (as cited in Gell and Coombs 1975): A drop or two of antigen is layered above a similar volume of undiluted antiserum, and in a positive reaction a ring of precipitate forms at the interface. This type of test is more sensitive for the detection of antigen than for antibody.

Precipitation techniques have been standardized and quantitated. A sensitive and reproducible method for the detection of antibody, which can be completed in less than 2 h, was described by Raney and McLennan (1979). To test their procedure, samples of rabbit antisera produced against a nucleoside-protein conjugate were added to tubes containing increasing amounts of this antigen in a buffered solution of 2% polyethylene glycol. The tubes were incubated at 37°C for 30 or 60 min and stored at 4°C for 15 minutes to 1 week with daily resuspension of the precipitates. The precipitate was washed and dried, and the protein content was determined at various time points. These results were compared with those of duplicate samples that were assayed by the standard procedure that does not incorporate the use of polyethylene glycol. In comparison with the standard procedure, the method of Raney and McLennan requires less time for complete precipitation (2 h vs 2 to 10 days); it is also more sensitive and more reliable.

Another precipitation technique, *gel diffusion*, has become widely used for the qualitative detection of antibodies to soluble antigens, and, though slightly slower than the ring test, it is more versatile. In single diffusion one reagent is incorporated in the supporting gel while the other, in liquid form, diffuses into it.

A quantitative modification of this procedure, *single radial diffusion* (SRID). was developed by Mancini et al. (1965). The technique, useful for quantitative determination of different immunoglobulins in serum, is performed as follows (Ouchterlony and Nillson 1973): a melted 3% agar solution is mixed with an equal volume of antiserum that has been properly diluted. The agar mixture is then poured as a 1-mm thick layer onto a glass plate. Holes 2 mm wide are punched in the gel and filled with accurately measured volumes of the antigen solutions to be tested along with references of known concentration of antigen. The plate is incubated in a humid atmosphere until the resulting precipitate becomes stationary. The area of the halo of precipitate is determined. Quantitation is possible since the area of the halo is directly proportional to the concentration of antigen. The "reversed" SRID technique allows quantitative determination of specific antibody when the antigen constitutes the internal reactant and the antiserum to be tested constitutes the external reactant.

The SRID technique is not very sensitive but can be used satisfactorily when high antibody titers are present, as in the secondary response to an antigen (Vos 1977).

Immunodiffusion methods have been employed in immunotoxicology studies for the assessment of antibody formation and for the measurement of immunoglobulins. Vos et al. (1973) measured serum tetanus-antitoxin levels in immunized guinea pigs using a RID procedure in which the tetanus toxoid was incorporated into the agar and the antiserum was allowed to diffuse out from the well. A slight suppression of the humoral immune response by sublethal doses of TCDD was detected with this method.

In a similar study, Luster et al. (1979), using a RID assay, quantified serum IgG in the serum of guinea pigs that had been exposed to TCDF. A minimal depression in serum IgG was observed, which correlated with antibody production to bovine gamma globulin.

The RID assay was used by Loose et al. (1979) to measure serum IgG, IgA, and IgM immunoglobulin concentrations in mice that had been treated with polychlorinated biphenyls or hexachlorobenzene, then immunized with SRBC. There was a reduction in serum IgA, IgG, and IgM that was concomitant with a decrease in the spleen plaque formation response. In the response to a secondary challenge only IgA was depressed. The chemicals were, however, administered at subtoxic levels.

Precipitation methods are simple and convenient, especially since commercial plates have been introduced. There seems to be a good correlation between antibody and Ig levels measured by SRID and antibody production measured by agglutination or plaque formation.

Hemagglutination and hemolysis. The direct agglutination test is another classical serological reaction which involves, simply, the clumping of a cell suspension by specific antibody (Gell and Coombs 1975). The test is performed by mixing dilutions of antiserum with the cell suspension and observing agglutination of the cells. Antibody combines rapidly with antigen, but no agglutination occurs until the cells come in contact with each other. Early agglutination tests were performed in tubes. A more efficient micromethod of the test was described by Stavitsky (1954) for use with tannic acid and protein-treated cells (see below); the test is also quite useful with untreated red blood cells. A modification of the method, one of several procedures recommended by White et al. (in press) for assessing the effects of toxicants or immunocompetence, will be outlined below. By adding complement to the system, the hemagglutinin reaction can be converted to a hemolytic reaction (Vos 1977). There is generally good correlation between the hemagglutination and hemolysis titrations.

In the method described by White et al. (unpublished observations), mice are exposed to the test chemical for the desired length of time and on day 0 are immunized intraperitoneally with 1 x 10^9 SRBC. On day 7, blood is collected in an anticoagulant (sodium citrate), and following centrifugation an aliquot of the plasma is removed and heat inactivated at 56°C for 30 minutes. Serial 1:1 dilutions are made in phosphate-buffered saline in conical microtiter plates to a final volume of 0.1 mL per well. Eight samples can be diluted at once using a semiautomatic technique. One-tenth milliliter of a 0.5% SRBC suspension is added to each well, and the plates are covered and placed in a 37°C humidified incubator. After 2 h the plates are observed for agglutination using a magnifying mirror. The end point is the serum dilution at which no agglutination occurs. (The titer is expressed as the reciprocal of the highest dilution that gives a positive reaction.) The secondary antibody response can also be tested in this way, and estimation of the proportions of the IgM and IgG antibodies can be achieved by degradation of IgM with 2-mercaptoethanol.

The sheep erythrocyte hemagglutination and/or hemolysin techniques have been used frequently to assess the humoral immune response following exposure of experimental animals to chemicals, such as trichloroethylene in mice (Sanders et al. 1982); PCB in monkeys (Thomas and Hinsdill 1978), carbon dust in mice (Zarkower and Morges 1972), and cigarette smoke in mice (Esber et al. 1973; Nulsen et al. 1974, Holt et al. 1976).

Sanders et al. (1982) detected slight suppression in the antibody response to SRBC following exposure of mice to subtoxic levels of trichloroethylene. Also, Thomas and Hinsdill (1978) observed suppression of the SRBC, but not the tetanus typhoid, antibody response of monkeys after exposure to PCB. They speculated that the SRBC response is more sensitive to damage than the tetanus toxoid response.

The hemagglutination and hemolysin assays are thus sensitive, easy to perform, economical, and quantitative. They can be used to titrate primary and secondary antibody responses and to measure the relative amounts of IgG and IgM produced in response to an antigen.

Passive hemagglutination. The chief difference in the precipitation reaction and the hemagglutination reaction is that in the former case the antigen molecules are free in solution and in the latter they are fixed on the surface (membrane) of the cell (Coombs and Gell 1975). Agglutination is far more sensitive than precipitation in detecting the antigen-antibody reaction (Herbert 1973, Coombs and Gell 1975). Boyden (1951) thus developed a technique for the adsorption of various proteins on the surface of tannic acid-treated (tanned cell technique) SRBC and demonstrated the agglutination of such cells by highly diluted specific antisera. The term "passive" agglutination is used because the red cells act as passive carriers of the protein antigens. Stavitsky (1954) adapted the technique to the titration of antitoxin and other protein sera using the microtiter assay previously discussed.

Technical details for the preparation of the antigen-coated cells for use in agglutination procedures have been outlined by Herbert (1973). These will be briefly summarized here:

Choice of Cells - Although it would seem logical to use cells of the same species for agglutination as that from which the antiserum is derived, most investigators use SRBC or human O cells. Both of these are fairly easy to obtain and have been satisfactory in the tanned cell technique, human cells being the more sensitive of the two. The cells should be fresh.

Preservation of Cells - Unless the fresh cells can be used right away they must be preserved (or fixed). This can be done before or after coating but usually takes place prior to coating because fixation may alter the coating antigen. The most commonly used fixative is formalin. Preserved cells are, however, considerably less sensitive than fresh ones.

Adsorption of Antigen by Red Cells - Coating of the red cells with antigen may be accomplished in three ways: by direct adsorption of antigen by the cells, by the tanned cell technique, and by chemical bonding of antigen to the cell.

Direct Adsorption - Direct coating of fresh cells can be done by simply incubating the antigen and cells at 37°C for approximately 2 h. Such cells are used in both hemagglutination and, with the addition of complement, hemolytic tests. These cells are, however, very insensitive to antibody in comparison to tanned cells. Preserved (fixed) cells can also be coated this way but with a longer incubation period, with constant mixing, and at the pH that is appropriate for the particular antigen.

Certain haptens can also become directly attached to red blood cells, and hemagglutination tests with such cells appear to be quite sensitive.

Tanned Cell Technique - It is generally assumed that tannic acid acts on red cells in such a way to cause them to take up protein antigens, but Herbert (1973) points out that the chief function may well be to increase the instability of the cells, making a normally nonagglutinating reaction result in agglutination. Cells that have been treated with tannic acid will show nonspecific agglutination settling patterns, but the tendency to agglutinate may be balanced by adding normal serum as a stabilizer. The coated cells are thus highly sensitive to agglutination, in the presence of a very small amount of antibody.

The tanning procedure itself is easy to perform and involves simply incubating the fresh or fixed cells with tannic acid in buffer for a brief period of time. The cells can then be stored for subsequent coating with antigen, which can be accomplished by incubating the cells and antigen at room temperature for a very short time. During the tanning and coating procedures the cells must be carefully washed at each step, the pH must be carefully monitored, and optimal concentrations of the reactants must be used. The cells thus prepared are ready for use in conventional titration procedures.

Attachment of Antigen to Red Cells by Use of an Intermediate Compound - Bis-diazotized benzidine is a suitable chemical for use in this procedure. Coupling the antigen to the cells is accomplished by simply incubating cells (usually formalinized), bis-diazotized benzidine, and antigen together for a short time and then washing the sensitized cells. Other chemicals recommended for this include chromic chloride, difluorodinitrobenzene, and carbodiimide.

Passive hemagglutination techniques have been employed to test the humoral response to the following: (1) *E. coli* lipopolysaccharide following exposure of rats to lead acetate (Luster et al. 1978) and of mice to carbon (Zarkower 1972; Zarkower and Morges 1972); (2) human gamma globulin following exposure of rats to cadmium chloride (Jones et al. 1971); (3) diphtheria toxoid following exposure of guinea pigs to DDT (Gabliks et al. 1973); (4) *Brucella abortus* antigen following exposure of mice to methyl mercury (Spyker 1975); and (5) tetanus toxoid following exposure of monkeys to PCB (Thomas and Hinsdill 1978).

Luster et al. (1978) tested the humoral immune response to both sheep erythrocytes (SRBC), a thymus-dependent antigen, and *E. coli* lipopolysaccharide, a thymus-independent antigen, in mice exposed to lead acetate. The SRBC response was suppressed by lead while the lipopolysaccharide response was not, indicating an alteration of the T-, rather than the B-, lymphocyte population.

The reciprocal effect was demonstrated in the study reported by Spyker (1975). Mice were exposed to methyl mercury in utero and were challenged 14 months later with *Brucella abortus* antigen, a thymus-independent antigen, and SRBC. In this case B-cell function was impaired while T-cell function was unaffected. (No impairment was observed in the response to either antigen when mice were challenged at 4 months of age, indicating that impaired immune function may be a delayed effect of prenatal exposure to methyl mercury.)

In summary, agglutination methods are objective, quantitative, sensitive, and versatile. Passive hemagglutination techniques are a bit complicated, but they are valuable tools for the study of responses to soluble antigens. Micromethods simplify the procedures and allow the use of small quantities of reagents, thereby lowering the cost of the tests.

Enzyme-linked immunosorbent assay. Another test that, similar to the radial immunodiffusion technique, has the capability for the measurement of both antigen and antibody in sera is the enzyme-linked immunosorbent assay (ELISA), which was introduced by Engvall and Perlmann in 1971. The technique was adapted by Vos et al. (1979a, 1979b, 1979c) for use in studies on the humoral immune response of rats. The following "sandwich" ELISA was used by them for the measurement of rat IgG and IgM:

Polystyrene tubes were coated (by rotation for 18 h) with antimmunoglobulin solution; test and reference serum were then diluted and added to the appropriate tube and the tubes were incubated under rotation for 1 h. After washing, 1 mL of a conjugate of horseradish peroxidase and antiimmunoglobulin (IgG or IgM) was added. The tubes were then incubated under rotation for 2 h. Excess conjugate was removed, and 1 mL of specific hydrogen peroxide substrate was added. Extinction was measured at 490 nm following incubation for one hour at room temperature. Vos et al. (1979a) reported that the measurements of IgM and IgG correlated well with results obtained by SRID. ELISA is less time-consuming than the SRID technique, and the macro-ELISA used here required less serum.

Vos et al. (1979a) also utilized ELISA for determining antibody titers to *E. coli* lipopolysaccharide and tetanus toxoid. The method performed and described by them was that of Ruitenberg et al. (1975):

Polystyrene microplates were coated with the antigens for 2 h at 37°C (LPS did not attach well and special procedures were required here). The microplate trays were incubated with 100 μL of test or control serum dilutions for 1 h at 37°C. After washing, 100 μL of the horseradish peroxidase-antiimmunoglobulin conjugate was added, and the microplates were incubated and rotated for 2 h. The plates were washed and substrate was added. After incubation for 1 h at room temperature the reaction product was evaluated visually and expressed as ^{2}log of the highest dilution giving a positive reaction. The ELISA results were compared with those obtained with passive hemagglutination, and ELISA was found to be more sensitive except when secondary response titers to tetanus toxoid were compared.

Vos et al. (1979b, 1979c) included the ELISA with routine studies to assess immune function in rats exposed prenatally and postnatally, or as adults, to hexachlorobenzene. The procedure appeared to provide a satisfactory assessment of the humoral immune response in the rats.

According to Vos et al. (1979a) and Koller (1982) the procedure is a reliable, sensitive, specific, and economic method for detecting IgM and IgG antibodies and antigens. It has the potential for large-scale screening because mechanization of all steps of the assay is feasible. In the laboratory of Koller (1982), ELISA has been accepted as the test procedure of choice for assessing humoral immunity in animals exposed to chemicals.

3.2.2.3 Other tests of B-cell function

B-cell function can be evaluated by procedures that measure parameters other than direct antibody formation. Two of these, the EAC rosette technique and transformation by B-cell mitogens, are discussed.

EAC rosette technique. There is a population of lymphoid cells that is capable of binding "erythrocyte-antibody-complement" (EAC) to form rosettes. This is possible because B-cell membranes possess a receptor for the third component of complement (C3).

Erythrocytes are prepared for this test by first reacting them with rabbit antierythrocyte antibody (forming the complex EA) (Bianco et al. 1970). The EA complex can then be treated either with serum from mice genetically deficient in C3 or with purified human complement components. The complex thus formed by antibodies, erythrocytes, and complement is the EAC complex.

Koller and Brauner (1977) applied the EAC rosette assay to the enumeration of B-cells from mice that had been exposed to lead acetate or cadmium chloride. In their experiment, spleen lymphoid cells were incubated with EAC for 5 min at 37°C; the cells were then centrifuged for 5 min and incubated on ice for 1 to 2 h. After gentle resuspension, the cells were stained with crystal violet and examined microscopically in a hemacytometer. Cells with three or more bound erythrocytes were counted as EAC rosettes. Fewer rosettes were formed by the B-cells from mice exposed to the chemicals than by B-cells from control mice. Cell viability tests indicated that this effect was not due to toxicity of the lead and cadmium.

The rosette test is a simple assay for the enumeration of B-cells and is one of the tests routinely employed by Luster and Faith (1979) for the detection of alterations in the humoral immune response.

Transformation of lymphocytes by B-cell mitogens. The technical aspects of the mitogen-stimulated lymphocyte transformation assay have been discussed in Section 3.2.1.2. The procedure is the same for both T- and B-cell transformation with the exception of the mitogens used. Bacterial lipopolysaccharides (LPS) are generally used to stimulate the mitogen response of B-cells, while concanavalin A and phytohemagglutinin are primarily T-cell mitogens. Pokeweed mitogen, which stimulates both T- and B-cells, is also used frequently.

The B-cell response has been shown to be sensitive to the immunosuppressive effects of metals (Gaworski and Sharma 1978, Koller et al. 1979), TCDD (Sharma et al. 1978), and TCDF (Luster et al. 1979), but the response has been either enhanced or unaffected by hexachlorobenzene (Vos et al. 1979b, 1979c).

3.2.3 Assessment of Indirect Parameters of Immunity

A number of indirect factors must be considered when evaluating the immune response. Indirect parameters commonly assessed include macrophage functions, hormones, complement, host resistance to infection, resistance to tumor challenge, and endotoxin hypersensitivity (Luster and Faith 1979). Of these, macrophage functions and host resistance to infection are often assayed in immunotoxicology studies. Some procedures for evaluating these parameters are discussed in the following sections.

3.2.3.1 Macrophage functions

Polymorphonuclear and mononuclear phagocytes are the two major classes of cells that are characterized by the ability to ingest and destroy invading microorganisms (van Furth et al. 1978). This process of phagocytosis is completed in the following stages: opsonization of the particles (viable and nonviable) by serum factors, attachment of the opsonized particles to the cell surface, engulfment of such particles, intracellular killing of microorganisms, and digestion of microorganisms and other ingested matter. Of these stages, ingestion and intracellular killing are most frequently tested.

Experiments for the study of macrophage functions can be performed in vivo or in vitro. In vitro methods are preferred by some investigators (Vos 1977, van Furth et al. 1978, Luster and Faith 1979). The advantages of these methods are that they allow the use of homogeneous cell populations so that the functions of individual cell types (e.g., monocytes, granulocytes, and macrophages) can be studied and they eliminate the effects of serum and other factors on the macrophage function.

The main disadvantage is that phagocytosis and intracellular killing do not appear to be extremely sensitive to the chemicals administered systemically in studies on the immune response. For example, in a few of the experiments reviewed, even though other immune parameters were altered by exposure to trichloroethylene (Sanders et al. 1982) or hexachlorobenzene (Vos et al. 1979b, 1979c) macrophage functions were not affected. Methods for obtaining macrophages for study have been reviewed in detail by Stuart et al. (1978) and van Furth et al. (1978).

Ingestion. Peritoneal macrophages of mice are frequently used in the study of phagocytosis. In the procedure described by van Furth et al. (1978) phagocytes and an equal number of bacteria are mixed together in a tube containing serum. The mixture is incubated at 37°C under continuous rotation. At various time points (0, 30, 60, 90, and 120 min) samples are taken from the suspension and centrifuged, and supernatants of the samples are plated on agar for subsequent bacterial colony counts; or the cell suspensions are examined microscopically after 15 minutes incubation for morphological assessment of phagocytosis. If colony counts are made, phagocytosis is measured by the decrease in the number of extracellular organisms in the supernatants. If morphological assessment is made, cells are dried on a slide and stained, and cells that have ingested bacteria are counted.

Sanders et al. (1982) describe an alternative method for assessing phagocytic activity in which peritoneal exudate cells are collected and counted and allowed to adhere to a 24-well Costar culture dish. The adhered cells are washed, and chromated SRBC (opsonized with IgG) is added. The plates are incubated and removed at 10 to 45 min. The cells are washed and scraped from the dishes and placed in tubes for gamma counting.

Intracellular Killing. Intracellular killing by peritoneal macrophages from the mouse can be assessed by the following method (van Furth et al. 1978). The cells can be removed and incubated with bacteria in vitro for 30 min, or the mouse can be injected intraperitoneally with the bacteria. Macrophages can ingest bacteria in vivo in 3 min. After the initial period of phagocytosis, the macrophages are washed, suspended in medium, and counted. The suspension is divided into aliquots, which are incubated at 37°C (one at 4°C). After 15, 30, or 60 min of incubation, aliquots are removed, the cells are disrupted by freezing and thawing, and the mix is plated on agar. The number of viable bacteria is determined from the number of colonies.

The assays just described are performed with peritoneal macrophages from mice. However, similar tests can be performed with alveolar macrophages. In addition, macrophages can be obtained from other species such as the rat and guinea pig. Clearance and intracellular killing by alveolar macrophages appear to be sensitive indicators of injury by inhaled gases and particles such as nitrous oxide (Acton and Myrvik 1972, Vassallo et al. 1973); cigarette smoke (Rylander 1971), ozone (Goldstein et al. 1971), and carbon black (Rylander 1969).

3.2.3.2 Host resistance to infection

The assessment of host resistance to infection following exposure to chemicals represents a practical approach to defining immunosuppression. For testing host resistance *Listeria monocytogenes* and *Streptococcus pneumoniae* are recommended because resistance to these organisms appears to be dependent primarily upon normal T-cell and B-cell functions, respectively (Vos 1977, Luster and Faith 1979). All phases of the cell-mediated immune response, including macrophage killing in the effector stage, participate in the resistance to *Listeria* (Vos 1977).

Tripathy and Mackaness (1969) first described the *Listeria* procedure and applied it to testing the effects of several drugs on the immune response. The assay can be performed on mice and is based on the principle that effective suppression of the immune response by drugs or other chemicals (which can be administered at the onset of infection or during the course of the infection) would result in continued multiplication of *Listeria*, particularly in the liver and spleen. The animals are injected intravenously with the live bacteria, and resistance can be measured in two ways: (1) mortality rates of treated animals are compared with those of the controls or (2) bacterial growth in the spleen is enumerated at days 4 and 6 after inoculation, when cell-mediated immunity interrupts the growth of the organism in vivo. The spleen is homogenized, and the homogenate is plated for the subsequent enumeration of colonies formed by viable bacteria.

The advantages of the technique as listed by Tripathy and Mackaness (1969) are as follows:

1. the immunosuppressive effect is quickly revealed
2. the method provides an accurate and quantitative assessment of the effect of chemicals.

When *Streptococcus pneumoniae* is the infectious agent, resistance to infection is simply assessed by comparing mortality rates of controls to those of treated animals (Vos 1977).

Numerous alternatives to the use of these organisms include the use of viruses and other bacteria and parasitic expulsion. Increased mortality due to impaired host resistance to infectious organisms has been demonstrated in various laboratory animals treated with chemicals such as PCBs (Friend and Trainer 1970, Thomas and Hinsdill 1978, Loose et al. 1979), metals (Exon et al. 1975, Gainer 1977, Koller 1979b, Exon et al. 1979), and TCDD (Thigpin et al. 1975).

3.3 ALLERGIC REACTIONS TO ENVIRONMENTAL CHEMICALS

Coombs and Gell classified allergic reactions in 1963. A detailed discussion of these reactions can also be found in Coombs and Gell (1975). The four classifications are briefly described in Table 3.5 and are illustrated in Figure 3.3. Coombs and Gell stress that the circumstances in which these four basic types of reactions may be studied in an uncomplicated form are limited and that the pattern seen in any one human disease is often complex, perhaps involving several of the responses listed in Table 3.5.

The Type I reaction in man includes both general and local anaphylaxis. In generalized anaphylaxis if the patient does not die rapidly, local symptoms develop such as bronchial asthma, hay fever, pulmonary edema, and urticaria. An example of local anaphylaxis is the appearance of a wheal at the site of antigen application, as in the prick or scratch diagnostic skin tests.

The Type II reaction is manifest as: complement-dependent antibody cytotoxicity (transfusion reactions, hemolytic disease of the newborn, or

Table 3.5. Definition of the Four Types of Allergic Reaction

I (Anaphylactic, reagin-dependent)

Initiated by allergen or antigen reactng with tissue cells (basophils and mast cells) passively sensitized (allergized) by antibody produced elsewhere, leading to the release of pharmacologically active substances (vasoactive amines). An example of local anaphylaxis is the appearance of a wheal at the site of antigen application, as in the prick or scratch diagnostic skin tests

II (Cytotoxic or cell stimulating)

Initiated by antibody reacting with either (1) an antigenic component of a cell or tissue element or (2) an antigen or hapten which has become intimately associated with these; damage may then occur in the presence of complement or of certain kinds of mononuclear cells. Stimulation of secretory organs may also occur (e.g., the thyroid). The Type II reaction is manifest as transfusion reactions, hemolytic disease of the newborn, or lesions produced in tissues by action of antibody and complement

III (Damage by antigen-antibody complexes)

Initiated when antigen reacts in the tissue spaces with potentially precipitating antibody, forming microprecipitates in and around the small vessels, causing damage to cells secondarily, or being precipitated in and interfering with the function of membranes, or when antigen in excess reacts in the blood stream with potentially precipitating antibody, forming soluble circulating complexes which are deposited in the blood-vessel walls or in the basement membrane and cause local inflammation or massive complement activation. Antigen excess complexes mediate allergic damage of three general kinds: (1) the Arthus reaction (situations in which antigen is in excess locally in the body); (2) serum sickness (antigen-antibody union takes place in the bloodstream and complexes are deposited at sites throughout the body); (3) "massive complement activation" (especially associated with endotoxin shock)

IV (Delayed, tuberculin-type, cell-mediated)

Initiated essentially by the reaction of actively allergized lymphocytes, probably of the T (thymus-derived) population responding specifically to allergen by the release of lymphokines, and/or the development of cytotoxicity without the participation of free antibody. Locally, it is manifested by the infiltration of cells at the site where the antigen is injected

Source: Adapted from Coombs and Gell (1975).

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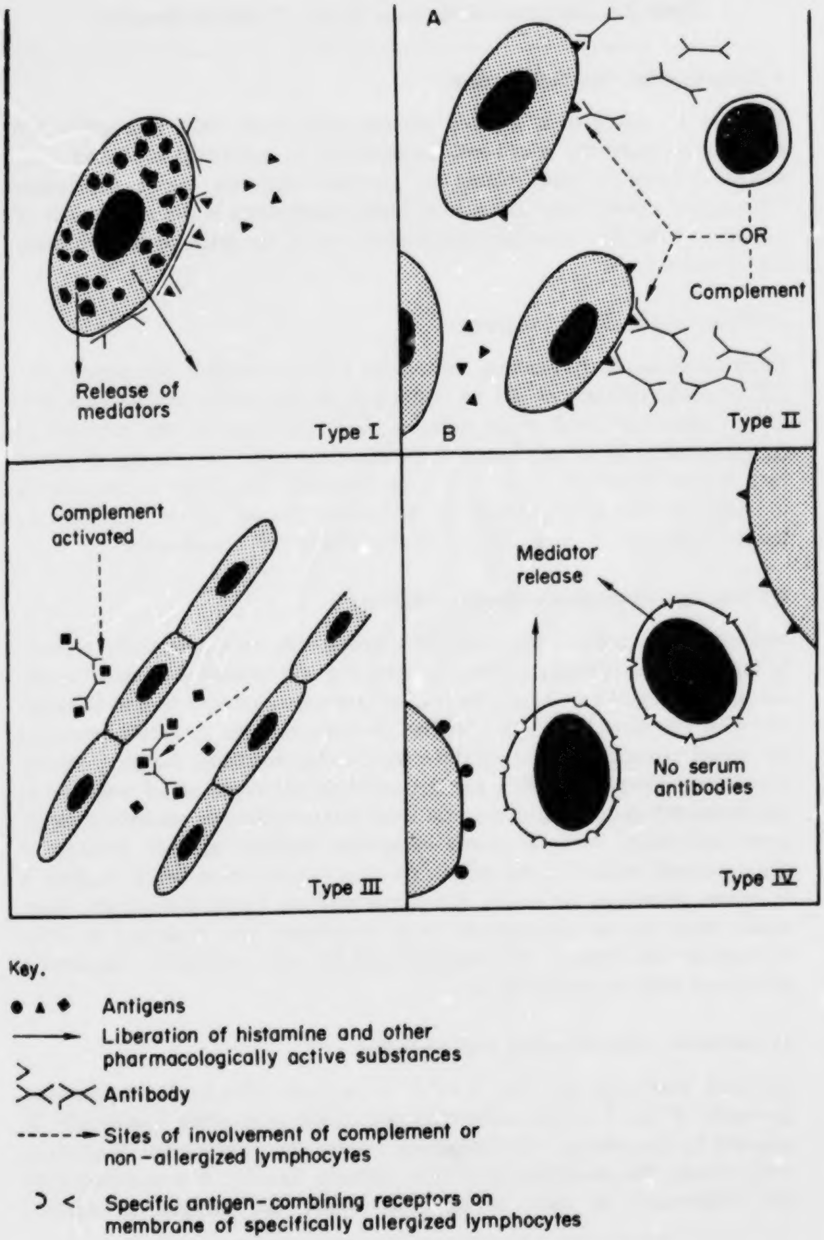

A
Release of mediators
Type I
OR
Complement
B
Type II
Complement activated
Type III
Mediator release
No serum antibodies
Type IV
Key.
● ▲ ◆ Antigens
→ Liberation of histamine and other pharmacologically active substances
Antibody
-----→ Sites of involvement of complement or non-allergized lymphocytes
Specific antigen-combining receptors on membrane of specifically allergized lymphocytes

Figure 3.3. The four types of allergic reaction. (Source: Coombs and Gell 1975.)

lesions produced in tissues by action of antibody and complement); cell dependent antibody cytotoxicity; antibody reacting with hapten or antigen combined with or adsorbed on tissue cells to cause cytotoxicity (drug sensitivity, e.g., purpura, hemolytic anemia, and granulocytopenia); antibody reacting with tissue cells to cause stimulation (thyroid stimulator, "enhancement" of malignant and other grafts).

The Type III reactions are characterized by antigen-antibody complexes formed in moderate antigen excess so that they do not precipitate but remain soluble, thus becoming toxic to the surrounding tissue (precipitated complexes are normally nonirritating and are easily cleared by phagocytes). Antigen excess complexes mediate allergic damage of three general kinds: (1) the Arthus reaction (situations in which antigen is in excess locally in the body); (2) serum sickness (antigen-antibody union takes place in the bloodstream, and complexes are deposited at sites throughout the body); and (3) "massive complement activation" (especially associated with endotoxin shock).

Type IV reactions are referred to as delayed hypersensitivity, cellular hypersensitivity, cell-mediated allergy, and others. Nomenclature is constantly under discussion. An essential characteristic of delayed reactivity is that the state can be transferred by cells although not by serum. It is currently thought that the cell-mediated response is a property of T-cells and has no need for B-cells. The development of the delayed lesion was described in Section 3.2.1.

Chemical allergens found in industrial environments usually affect a small subset of workers who are hypersusceptible to the low-dose exposure legally permitted in chemical plants (Adkinson 1977). For example, from 1 to 10% of industrial workers exposed to legally permissible levels of organic isocyanates will develop hypersensitivity reactions following low-dose exposure to the chemicals (Pepys 1976). Similarly, a small group of highly susceptible workers becomes immunologically sensitive when exposed to numerous other industrial chemicals, and in almost all cases the principal allergen has been identified as a chemical that is highly protein-reactive (Adkinson 1977).

Landsteiner and Jacobs (1935, 1936) were the first to demonstrate the antigenicity of chemicals. They induced reproducible immunological effects in guinea pigs with simple compounds, halogenated dinitrobenzene derivatives. They discovered, however, that if the halogen was replaced with a hydrogen, hydroxyl, methane, or an amino group the compound was immunologically inactive. The ability to sensitize seemed to be related to the lability of the Cl ion when treated with alkali. The investigators concluded that this property facilitated the binding of a simple nonantigenic chemical with an endogenous protein carrier, creating an antigenic complex.

This concept led to the "hapten theory," named for the chemical or "hapten" or "fastening agent" involved (Loomis 1974). A hapten is, therefore, a compound of low molecular weight that combines with a protein carrier to form a product having antigenic properties that can elicit antibodies in the animal. Although many chemicals do not appear to react with protein in vitro, these complexes are nonetheless formed in the intact animal, probably through metabolic alteration of the chemical.

It is now known that simple chemicals of molecular weight less than 1000 cannot elicit an immune response unless they are capable of covalent linkage to carrier molecules (Adkinson 1977).

3.3.1 Allergic Response to Inhalants

The lung is constantly exposed to biologically active materials in inhaled gases and particulate matter. The immune response to inhaled physical and chemical agents and the injurious consequences of this response have been reviewed in detail by Burrell (1977) and De Bruin (1976).

Burrell (1977) recognized the respiratory route as an excellent means of immunization, particularly in the stimulation of the IgA and IgE classes. He also recognized that these immune responses are not always beneficial to the host.

Inhalants thought to evoke allergic responses include physical and chemical agents and antigens of plant and animal origin. Some of these are listed in Table 3.6.

Illnesses due to inhalation of organic dusts include farmer's lung, bagassosis, byssinosis, ptilosis, tabacosis, malt worker's lung, and suberosis (De Bruin 1976). The organic dusts set up toxic asthma and allergic alveloitis, responses thought to be of the Arthus type. Illness due to inhalation of inorganic particles include inorganic pneumoconiosis and chronic pneumoitis. (The latter disorder is a consequence of a delayed hypersensitive reaction induced by exposure to beryllium.)

3.3.2 Allergic Response to Dermal Sensitizers

Many chemicals have been demonstrated to evoke allergic reactions of the skin. The most common response is allergic contact dermatitis, a delayed-type hypersensitivity reaction. Among these allergenic chemicals are: 2,4-dinitrochlorobenzene, *p*-nitrodimethylaniline, *p*-phenylenediamine, hexavalent chromium, nickel, beryllium, and cobalt.

For the induction of contact sensitization the eliciting contact allergen must be applied to the skin, penetrate the epidermis and become combined with protein, with which a complex antigen is formed (De Bruin 1976).

Contact sensitivity is apparently a complex hypersensitivity to numerous conjugates (formed in situ) of a chemical with various analogous proteins.

3.3.3 Tests to Detect the Sensitizing Potential of Chemicals

Marzulli and Maibach (1977) stated that three types of tests are needed to evaluate skin sensitization potential:

Table 3.6. Respiratory Allergens

- Physical agents
  - Asbestos
  - Coal dust
  - Silica
- Chemical agents
  - Beryllium
  - Detergent enzymes
  - Isocyanates
  - Miscellaneous chemical incitants
- Antigens of plant origin
  - Aspergilli
  - Cotton and other textile dusts
  - Miscellaneous hypersensitivity pneumonitis incitants
  - Thermophilic actinomycetes
- Antigens of animal origin

Source: Adapted from Burrell (1977).

1. the predictive test to identify allergenic substances
2. the diagnostic test to identify a substance that may be actually producing skin reactions, and
3. the use test to provide information regarding the safety of ingredients in combinations before they enter the marketplace.

Some of the predictive tests of skin allergy will be described in the following sections. These are useful for testing chemicals to which occupational exposure may occur via the skin. Also, a new test for the detection of allergy induced via respiratory exposure will be described.

3.3.3.1 Skin sensitization

According to the OECD (1981), skin sensitization tests require an initial exposure to a test substance, followed by a challenge exposure which is administered no less than a week later. Sensitization is evaluated from the reaction to the challenge exposure, usually 24 and 48 h after the pertinent exposure, based on the proportion of each group that became sensitized and the severity of the reaction in each animal. The recommended species for animal testing is the guinea pig, with the number and sex determined by the method used. For the induction exposure, the concentration used should produce a skin reaction; for the challenge exposure the concentration should be nonirritating.

Skin predictive test procedures in guinea pigs have been identified by Klecak (1977) for ascertaining the allergenic potential for chemicals. These include the Draize test, Freund's complete adjuvant test, the guinea pig maximization test, the "split adjuvant" test, the Buhler test, and the open cutaneous test.

The Draize Test - In this test, guinea pigs are sensitized intradermally on the flank every other day for 18 days, and skin reactions are read 24 h after each injection. The challenging dose is administered intradermally on the opposite flank on day 35. Controls are treated only with the challenging dose. On days 36 to 37 the test areas are shaved, and the intensity of erythema and occurrence and size of edema of the test reaction are recorded. The test and control reactions are compared. The test is difficult to quantitate but it is easy to perform, and material and operational expenditures are minimal.

Freund's Complete Adjuvant (FCA) Test - In the FCA test, guinea pigs are sensitized every other day for 10 days intradermally in the flank with a mixture of test material and FCA (killed mycobacteria). Skin reactions are read 24 h after each injection. The challenging doses of appropriate concentrations are applied topically to the opposite flank. The challenging dose is not mixed with Freund's adjuvant. Skin reactions are evaluated as for the Draize test.

The technique is simple and inexpensive, but Klecak (1977) believes that it is more useful for identifying sensitizers than as a predictive test.

The Guinea Pig Maximization Test - For this test, guinea pigs are immunized on the shoulder both intradermally with the test agent in FCA and topically (test agent in petrolatum) 7 days later. The challenging dose is applied topically to the flank on day 21, and the reactions are read on days 23 and 24. This test combines the factors that favor sensitization in the guinea pig and is considered to be the most sensitive procedure for detecting allergenic potential in these animals. There is also a high degree of correlation between the results of this test and the human maximization test (Klecak 1977).

The Split Adjuvant Technique - This test makes use of the finding that intradermal challenge of FCA beneath the site of topical application of allergen potentiates sensitization. Sensitizing doses are administered to the back (to which dry ice has been applied) of the guinea pig on days 0, 2, 4, and 7. On day 4 FCA is injected intradermally twice into the sensitization site. The challenging dose is applied to the back on day 20, and the reaction is read on days 22, 23, and 24. Klecak (1977) believes this method is particularly effective with weak sensitizers, but it is time-consuming.

The Buhler Test - In this test, the chemical is applied to the flank and held there with an occlusive patch for 6 h on days 0, 7, and 14. On day 28 the challenging dose is applied to the same site for 24 h. The skin reaction is read on days 29, 30, and 31, and the results are compared with those of negative and/or vehicle controls. The test is fairly expensive, but it corresponds to the practical needs of a predictive test method (Klecak 1977).

The Open Epicutaneous Test - In the open epicutaneous test, guinea pigs are immunized on the flank three or five times weekly for four weeks, always using the same site, with undiluted compound and/or its progressive dilutions. The reactions are read once a week or 24 h after each application. On days 21 and 35 the minimal irritant (previously determined) and some lower concentrations are applied to the contralateral flank. A concentration is considered to be allergenic when at least one animal in a group shows a positive reaction with nonirritant concentrations. The test is suitable for testing simple chemicals as well as finished products, and the topical application methods used are similar to common use (Klecak 1977).

Technical details and original references for the skin sensitization tests can be found in Klecak (1977).

3.3.3.2 Respiratory sensitization

Although experimental induction of respiratory hypersensitivity has been readily accomplished with natural antigens (such as animal danders and pollen) and with purified proteins, the induction of such a response with hapten-protein complexes has been more difficult (Karol et al. 1978).

Karol et al. (1978), however, were successful in producing respiratory hypersensitivity in guinea pigs with repeated exposure to aerosols of hapten-ovalbumin conjugates. The animals were exposed to aerosols of *p*-azobenzenearsonate ovalbumin and *p*-tolylureido ovalbumin in a Plexiglas inhalation chamber. Respiratory hypersensitivity was evaluated on the basis of changes in respiratory rate, a sensitive indicator of the response. The hypersensitivity response was hapten-specific, as were most of the antibodies produced during the course of the study. The guinea pig model was used in a later study in which respiratory hypersensitivity was induced with hexyl isocyanate-ovalbumin aerosol (Karol et al. 1979). Hapten-specific antibodies were detected as well as hapten-specific IgE antibodies. Karol et al. (1978) recommend their method for screening industrial chemicals for their potential induction of respiratory hypersensitivity. In more recent studies using the guinea pig model, Karol (1983) and Karol et al. (1981) demonstrated that the immunologic response to toluene diisocyanate following inhalation exposure is dose dependent and that respiratory tract hypersensitivity can be induced by dermal contact.

3.4 TIER TESTING

Tests suitable for assessing the various cellular and humoral functions of the immune system have been discussed in the previous sections. If the evaluation of immunocompetence is to become a permanent fixture in routine toxicity test protocols, then the selection of the proper combination of tests for use in these protocols becomes an important consideration. First, it seems obvious that both humoral and cellular functions should be tested. Second, it is important that tests be chosen that will efficiently and economically provide maximum information.

Investigators in the field of immunotoxicology have made their independent recommendations for the types of immunology procedures to be included in routine toxicity studies or in tier testing protocols. Suggestions of Moore and Faith (1976), Luster and Faith (1979), Speirs and Speirs (1979), Dean et al. (1979b), White et al. (unpublished observations), and Luster et al. (1982b) are presented in the following sections.

3.4.1 Moore and Faith (1976)

Moore and Faith, interested in the developing immune system, recommended the rat and mouse as the species to be tested, initially at weaning. The following methods were selected because they were generally accepted by immunologists and were within the capabilities of many laboratories.

A. Tests for humoral (or B-cell) immune function
  1. Hemagglutination techniques using
    a. lipopolysaccharide (thymus independent)
    b. SRBC (thymus dependent)
    In a minimal experimental design where only one antigen can be tested, one should select the SRBC test because both T- and B-cells can be evaluated with this procedure.
  2. Mitogen stimulation of thymus and spleen cells with lipopolysaccharide (a B-cell stimulator)

B. Tests of cellular (or T-cell) immune function
  1. Radiometric ear test
  2. Mitogen stimulation of thymus and spleen cells with phytohemagglutinin and Concanovalin A, T-cell stimulators.

Moore and Faith further recommended that:

1. Chemicals that depress immune function in adults should be evaluated in the fetus/neonate.
2. Routine teratology studies should include an evaluation of the thymus and spleen (organ weights and histologic examination). If effects are observed on maturation of the tissue, then one should consider subsequent experiments that specifically assess the immune response.

3.4.2 Luster and Faith (1979)

These investigators outlined the immune tests used in their laboratory at the National Institute of Environmental Health Sciences. The initial assessment of the effects of a particular chemical on the immune system is generally made in a routine toxicity study. At necropsy Luster and Faith perform the following procedures:

A. Examination of lymphoid organs (thymus, spleen, lymph nodes, and bursa of Fabricius) and perhaps the adrenals
   1. Gross examination
   2. Histopathology
   3. Lymphoid organ to body weight ratio
   4. Quantitation of viability for the determination of cytotoxicity

B. Hematology
   1. Differential
   2. Leukocyte count

C. Immunology
   1. Serum immunoglobulin concentrations—electrophoresis
   2. Lymphocyte transformation (PHA and LPS)
   3. T-cell rosettes (not practical in mice or rats)
   4. Surface antigens on lymphocytes (theta antigen in mice)

D. Clinical signs, infections, tumors

Further assessment of the immune system can be accomplished with the following:

A. Tests of cell-mediated immunity
   1. In vitro transformation of lymphocytes by concanavalin A
   2. In vitro mixed lymphocyte cultures
   3. Radiometric ear test
   4. Assay for migration inhibition factor

B. Tests of humoral-mediated immunity
   1. In vitro transformation of lymphocytes by dextran sulfate or pokeweed mitogen
   2. B-cell surface antigens
      a. EAC rosettes
      b. Fluorescent labeling of B-cell immunoglobulins

   3. Quantitation of serum immunoglobulins by radial immunodiffusion
   4. Antibody response to T-dependent and T-independent antigen using the plaque assay

C. Tests of indirect immunity

   1. Macrophage
      a. In vivo uptake of aggregated ^{125}I-BSA or colloidal carbon
      b. In vitro or in vivo determination of phagocytosis and intracellular killing of bacteria
      c. Cytotoxicity and proliferation
   2. Hormone concentrations
      a. Cortisol/cortisone
      b. Estrogens or androgens
   3. Complement (measurement of C-dependent hemolysis)
   4. Host resistance to infection
   5. Resistance to tumor challenge
   6. Endotoxin hypersensitivity

The tests were selected by Luster and Faith for their reliability and for their ability to detect subtle differences in immune status.

3.4.3 Speirs and Speirs (1979)

The Immunotoxicology Program at the National Center for Toxicological Research has devised a model for in vivo assessment of the effects of toxic agents on immunocompetence. This procedure is summarized as follows:

1. Immunize mice with an *antigenic mix* consisting of:
   Diphtheria toxoid
   Pertussis vaccine
   Polyvalent pneumococcal polysaccharide antigens
   Tetanus toxoid
2. Determine levels of antibody and other plasma proteins using radioimmunoassay procedures.
3. Assess functional capabilities of macrophages, lymphocytes, and other blood cells using cytochemical analysis.
4. Develop an *immune profile* for each toxicant tested. Compare with profiles of known immunosuppressants and other toxic agents.

5. Compare the *immune profiles* of mouse and man with respect to a particular toxicant.

In their model Speirs and Speirs (1979) use licensed vaccines for immunization, and commonly used pharmaceutical agents as prototype immunosuppressants so that animal and human data can be more readily compared. Risk assessment is based on comparison of immune profiles of animals treated with known immunotoxicants with those of animals treated with a potential toxicant.

3.4.4 Dean et al. (1979b)

This group at Litton Bionetics has been involved for the last few years in developing and refining assays of humoral and cell-mediated immunocompetence. They have proposed a tier approach for screening for immunological effects (tier I) and to help determine which mechanisms are responsible for the effects observed (tier II). The assays were selected on the basis of relevance to the human experience, cost, reproducibility, ease of performance, and application to routine toxicology studies. The tests included in tiers I and II are listed in Table 3.7.

After 90 days of a chronic or subchronic toxicity study, 15 animals are removed for immunology studies. Testing can then proceed in either a hierarchical or nonhierarchical fashion. Hierarchical testing assesses chemicals in a stepwise procedure following administration of a dose of the test agent with minimal or no overt toxicity. The testing begins with the simplest or least expensive assays and continues through the more time-consuming and expensive in vitro tests. It is assumed that those chemicals with deleterious effects would be detected with the initial assays and would require further testing only when information regarding mechanism is required.

In nonhierarchical testing the assays (tier I) would all be performed at once. This procedure is useful when the number of compounds to be tested is small or when the data are needed quickly.

If no immunological effect is observed in tier I, no further studies are performed. If a positive effect (suppression or enhancement) is observed, the tier II assays are performed.

3.4.5 White et al. (Unpublished Observations)

This group at the Medical College of Virginia has developed a procedure to investigate the effects of a given chemical on the immune system against a background of standard toxicological procedures.

An acute study to determine the LD_{50} is performed first. Next, a subchronic 14-day range-finding study is performed, usually at 1/10 and 1/100 the LD_{50}. The parameters measured in the 14-day subchronic study are:

Table 3.7. Testing Approaches for Evaluating the Immunobiologic Effects of Food Additives, Drugs, and Environmental Chemicals

	Tier I: Screening	Tier II: Mechanism
Clinical laboratory	Hematology Blood chemistry Urine	Specific blood or tissue levels of compound Hormone levels
Pathology	Lymphoid organs • Relative weight • Cell viability • Histology	Cell surface markers • T-cells • B-cells • Null cells • Fc-cells
Cell-mediated immunity	Delayed hypersensitivity (DHS) • T-dependent antigen (keyhole limpet hemocyanin, bovine gammaglobulin, tetanus toxoid) toxoid study using the radiometric assay Lymphocyte proliferation • T- and B-cell mitogens • Mixed leukocyte culture	DHS • T-independent antigen Lymphocyte proliferation antigen Helper cell function Macrophage function
Humoral immunity	Ig levels Specific antibody tier • T-dependent antigen Jerne plaque assay • T-dependent antigen	Mishell-Dutton assay Local product of antibody Specific antibody tier • T-independent antigen (SIII) Cytotoxicity
Susceptibility Immunepotentiation Tumor challenge	Tumor challenge - TD_{10-40} TD_{70-80}	Bacterial challenge Virus challenge

Source: Adapted from Dean et al. (1979b).

A. Standard toxicology

1. Body weights[b]
2. Necropsy—gross pathology[b]
3. Organ weights[b] (brain, liver, spleen, lungs, thymus, kidneys, and testes)
4. Hematology[b] (leukocyte counts, hematocrit, hemoglobin)
5. Clinical chemistries[a] (glutamic pyruvic transaminase, lactic dehydrogenase, blood urea nitrogen)
6. Blood coagulation[b]

B. Immunotoxicology

1. Cell-mediated immunity[c]—delayed-type hypersensitivity response to sheep erythrocytes (footpad assay)

2. Humoral immunity[a]—spleen antibody-forming cell response to sheep erythrocytes (IgM plaque-forming cells)

Three sets of mice are used; the superscripts a, b, and c designate the group on which the test is performed.

The 90-day subchronic study is then performed to demonstrate the target organ. The following variables are measured during and at the end of the 90-day study:

A. Standard toxicology
    1. Body weights, twice weekly
    2. Fluid consumption
    3. Necropsy[a]—gross pathology
    4. Organ weights[a]—brain, liver, spleen, lungs, thymus, kidneys, and testes
    5. Urinalysis—pH, glucose, protein, bilirubin, blood, and urobilinogen
    6. Hematology[a]—hematocrit, erythrocytes, leucocytes, differential, platelets, and hemoglobin
    7. Coagulation[a]—prothrombin time, activated partial thromboplastin time, and fibrinogen
    8. Clinical chemistries[b]—sodium, calcium, potassium, serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase, lactic dehydrogenase, total protein, albumin, creatinine, and blood urea nitrogen

B. Hepatic microsomal mixed functional oxidase parameters
    1. Liver weight
    2. Microsomal protein
    3. Glutathione levels
    4. P-450 content
    5. Cytochrome b5
    6. Aminopyrine N-demethylase
    7. Aniline hydroxylase

C. Cell-mediated immunity
    1. Delayed-type hypersensitivity response to sheep erythrocytes[b]
    2. Spleen cell response to Concanavalin A[b]

D. Humoral immunity
    1. Spleen antibody-forming cell response to sheep erythrocytes[b] (footpad assay)

  2. Spleen cell response to lipopolysaccharide[b]
  3. Basal levels of IgM, IgG, and IgA

E. Functional activity of the reticuloendothelial system

  1. Vascular clearance rate of sheep erythrocytes[d]
  2. Organ uptake of sheep erythrocytes[d]
  3. Chemotaxis, adherence, and phagocytosis of peritoneal exudate cells[e]

F. Bone Marrow[a]

  1. DNA synthesis
  2. Granulocyte—monocyte stem cells

Five sets of mice are used; superscripts a, b, c, d, and e designate the group on which a test is performed.

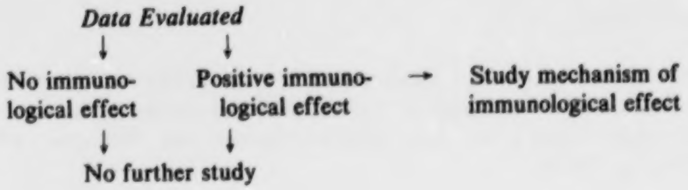
White et al. demonstrated that these assays, used to evaluate immune status, are quantifiable and reproducible and that such assays can be easily incorporated into a routine toxicology study.

3.4.6 Luster et al. (1982b)

In November 1979, the National Institute of Environmental Health Sciences held a Consensus Meeting to develop a list of relevant immunologic parameters suitable for evaluating chemically induced immunotoxicity. The group of more than 35 immunologists and toxicologists of varied experience and expertise prioritized the following scheme of immunologic parameters and methods considered necessary to measure immunotoxicity. These tests are to be performed in the order listed.

Chemical for immunological evaluation → *Chronic or Subchronic Study*
↓ ↓
Immunology Screening Panel

1. Pathotoxicology-hematology, liver, chemistry, serum proteins, lymphoid organ weights, and histology.
2. Host resistance-tumor challenge and infectious agent challenge.
3. Radiometric delayed hypersensitivity.
4. Lymphoproliferative responses-PHA, Con A, LPS, and MLC.
5. Humoral immunity-Ig levels, specific antibody titer, plaque-forming cell assay.
6. Macrophage function assays.
7. Bone marrow progenitor cell assays.


Data Evaluated
No immunological effect
Positive immunological effect
Study mechanism of immunological effect
No further study

3.5. SUMMARY

Reports of immunotoxic effects of environmental chemicals, some at clinically subtoxic levels, have stimulated interest in tests of immune function and in the inclusion of such tests in routine toxicity test protocols. These procedures provide information related to direct injury to the immune system and, equally important, are capable of detecting subtle alterations in the immune response. This system could be an extremely sensitive indicator of the general toxicity of certain chemicals.

The preceding review was undertaken to examine some of the test methods capable of detecting chemically induced alterations in the specific, as well as the nonspecific, immune response.

Tests of cell-mediated, humoral, and indirect immune functions were described, and examples of the use of these tests in the assessment of the potential immunotoxic effects of chemical were presented. A few tests that are useful for detecting the sensitizing effects of chemicals were also included.

There was no evidence to indicate that any particular test or battery of tests would be more suitable for evaluating a particular class of chemicals than others. However, there are indications that certain chemical classes may be selectively toxic to the humoral immune system, while others may affect cellular immunity. For example, lead, cadmium, mercury, polychlorinated biphenyls, and DDT impair primarily the humoral immune response, while organochlorines impair primarily the cell-mediated response. These observations, however, should not preclude the assessment, in toxicity testing, of both humoral and cell-mediated immunity.

Information related to the costs of these tests was not found in the literature. Generally, immunology procedures are regarded as being fairly expensive; but the introduction of in vitro techniques may enhance the possibility of large-scale testing at more reasonable costs.

Recommendations for the types of immunology tests that should be included in routine toxicity studies or in tier-testing schemes were presented.

3.6 GLOSSARY

ADJUVANT. A substance administered with or before an antigen that nonspecifically enhances or alters the cell-mediated or humoral immune response to that antigen (Herbert and Wilkinson 1971, Sharma 1981b).

ALLERGEN. An antigenic substance capable of eliciting an allergic response or an allergic state (Herbert and Wilkinson 1971, Sharma 1981b).

ALLERGENIC. Causing allergy (Sharma 1981b).

ALLERGY. (1) Hypersensitivity; (2) heightened reactivity to antigen, according to Gell and Coombs, which is synonomous with immunity (Herbert and Wilkinson 1971); (3) abnormal or harmful reaction which includes delayed, immediate, or contact hypersensitivity and may also include a number of reactions that have no immunological basis (Platts-Mills 1982).

ALLOANTIGEN. Cell-surface antigens in different strains of the same species (Wust, unpublished observations).

ALLOANTISERA. Mouse or rat antisera prepared by immunizing one inbred strain with cells from another strain (Wust, unpublished observations).

ALLOGRAFT. A syngeneic graft, or one that has been exchanged between two genetically dissimilar members of the same species, such as of two different inbred strains (Herbert and Wilkinson 1971).

ANAPHYLAXIS. An immediate hypersensitivity or Type I reaction resulting from the administration of antigen to a primed subject. The reaction is caused by the release of vasoactive substances, such as histamine and leukotrienes, when antigen combines with antibody on cell surfaces. The reaction may be generalized or localized. The systemic reaction can be violent and can cause death in minutes (Herbert and Wilkinson 1971; Wust, unpublished observations).

ANTIBODIES. Immunoglobulins that are produced in the body by cells of the lymphoid series, particularly plasma cells, in response to stimulation by antigen that are capable of specific combination with antigen (Herbert and Wilkinson 1971, Sharma 1981b).

ANTIBODY TITER. In serological reactions, a measure of units of antibody in an antiserum per unit volume of the undiluted serum. To determine the titer, the serum is serially diluted, antigen is added, and the end point is determined (Herbert and Wilkinson 1971).

ANTIGEN. A substance that elicits a specific immune response, either cell-mediated or humoral, when introduced into the tissues of an animal. An antigen may also induce specific immunological tolerance (Herbert and Wilkinson 1971). Can be a protein, nucleic acid, carbohydrate, or lipid (Wust, unpublished observations).

ANTIGEN PROCESSING. Macrophage-antigen interaction in which the macrophage localizes the antigen and brings it closer to the antibody-forming cell. This interaction may also result in enhanced immunogenicity of the antigen (Koller 1981).

ANTIGEN RECOGNITION SITE. The site on the surface of a lymphocyte which may be identical to an antibody-combining site. Can react specifically with antigen and thus initiate humoral or cell-mediated immune resposes (Herbert and Wilkinson 1971).

ANTIGEN-ANTIBODY REACTION. The interaction between an antigen and the antigen-binding receptor on the surface of a small lymphocyte which stimulates the differentiation and proliferation of the cell, leading to the production of a progeny of effector cells (which execute the immune response) and of memory cells (which are responsible for the enhanced or secondary response following a subsequent encounter with the same antigen) (Miller 1975). The immune system responds to an unlimited variety of antigens, some of them normally not present in nature (Polak 1977).

There is now evidence for the existence, on the cell membrane of small lymphocytes, of antigen recognition units that can bind antigenic determinants and of Ig molecules with specificity similar or identical to that of secreted antibody (Miller 1975). There is also evidence to show that the Ig determinants on the cell membrane are the antigen-binding receptors. Apparently both T- and B-cells have antigen-binding receptors on their surface, but they are of such low density on nonimmune cells that they cannot be detected by routine antigen-binding techniques.

ANTISERA. Serum, from an animal, that contains antibodies specific to a given antigen [e.g. anti-ovalbumin (Herbert and Wilkinson 1971)].

ARTHUS REACTION. An inflammatory reaction that follows the administration of antigen to an animal that already possesses precipitating antibody to that antigen. The reaction is caused by the formation, in the presence of complement, of antigen-antibody complexes that adhere to the endothelium of vessels and become surrounded by fibrin, platelets and neutrophils. This is followed by plugging of the vessels with thrombi and exudation of neutrophil-rich fluid into the surrounding tissues. The reaction is manifested as an ulcer that appears several hours after intradermal injection of antigen into a primed animal (Herbert and Wilkinson 1971).

B-LYMPHOCYTE. B-cells arise from stem cells in the bursa of Fabricius in birds and in the bursal equivalent in mammals (thought to be gut-associated lymphoid tissue or bone marrow) (Faith et al. 1980). B-cells mature independently of thymic influence (Koller 1979a). They circulate and rest temporarily in the thymus-independent areas of the spleen (follicles and peripheral regions of the white pulp) and lymph nodes (follicles and medulla) (Faith et al. 1980), where they constitute 10 to 20% of the nucleated free cells. They are not present in the thymus (Wust, unpublished observations).

B-cells are stimulated by antigen to become antigen-specific cells that proliferate and differentiate into plasma cells, which secrete antibody or become memory B-cells.

BACILLE CALMETTE-GUERIN (BCG). An attenuated, living bovine strain of *Mycobacterium tuberculosis* used to vaccinate against tuberculosis and leprosy (Herbert and Wilkinson 1971).

BLASTOGENESIS. Stimulation of cell (lymphocyte) blast formation by an antigenic substance (Sharma 1981b).

BLASTOGENIC. Capable of producing cell blasts. In immunology, antigenic substances capable of producing cell blasts (Sharma 1981b).

BONE MARROW. The principal source of the lymphoid stem cell in postnatal life and the only general hemopoetic tissue in the healthy adult. The vascular compartment of the bone marrow is occupied by the vessels that distribute nutrients throughout the marrow cavity and the hematopoetic compartments (red marrow), which contain erythrocytes, granulocytes, lymphocytes, megakaryocytes, monocytes, macrophages, plasma cells, mast cells, and their precursor stem cells. In adult animals much of the red marrow is replaced by fatty tissue and becomes yellow marrow. The bone marrow does not contain significant numbers of fully competent thymus-dependent lymphocytes. However, theta-positive incompletely developed T-cells and more mature T-cells are demonstrable. The marrow also contains significant numbers of B-lymphocytes (fully differentiated immunoglobulin-producing lymphocytes) and plasma cells (Bloom and Fawcett 1969, Herbert and Wilkinson 1971, Park and Good 1974).

BURSA-EQUIVALENT. A poorly identified tissue in mammals, considered to be equivalent to the bursa of Fabricius in birds, which gives rise to B-cells (Luster et al. 1982b). The bursa is a saclike lymphoepithelial structure arising as a dorsal diverticulum from the cloaca of young birds that contains many lymphoid follicles in which lymphopoiesis proceeds until the structure involutes about the time of sexual maturity (Herbert and Wilkinson 1971). The lymphocytes are B-cells that migrate from the bursa to colonize the secondary lymphoid organs (Wust, unpublished observations). The bursa is associated with humoral immunity, demonstrated by the fact that bursectomized chickens fail to make antibodies to a variety of antigens and lack plasma cells and germinal centers in their lymphoid tissues (Herbert and Wilkinson 1971).

CELL-MEDIATED IMMUNITY (CMI). The type of specific immunity that is effected by specifically sensitized lymphocytes and can be transferred with those cells. (Vos 1977, Faith et al. 1980). The main categories of cell-mediated immunity include: classical cell-mediated protective immunity, which is mainly effective against protozoal, viral, fungal, and some bacterial infections; delayed hypersensitivity skin reactions to extracts or whole suspensions of organisms, also identified as tuberculin hypersensitivity; chemical contact sensitivity; allograft rejection; immunological surveillance to tumors; and certain organ-specific autoallergic diseases, such as thyroiditis, encephalomyelitis (following rabies vaccination), adrenalitis, and orchitis (Turk 1975, Faith et al. 1980).

CELL SURFACE MARKERS. Antigens or alloantigens on the surface of lymphocytes (IgD and IgM on B-cells; theta and TL antigens on T-cells; and Ly alloantigens on both T- and B- cells) (Luster et al. 1982b).

CF. Chemotactic factor.

CHEMOTACTIC FACTOR. A product of sensitized T-lymphocytes exposed to antigens that direct the migration of leucocytes to sites of inflammation (Allison 1982).

CLINICAL ALLERGY. A disease or diseases in which hypersensitivity plays a part, such as contact hypersensitivity, hay fever, and asthma.

CLONAL THEORY. The theory of F. M. Burnet (1959), who believes that if a cell comes in contact with an antigen, and if any part of that antigen happens to fit the antigen-binding site of the cell, the cell is stimulated to divide and increase its production of that particular globulin. Because each region of a given antigen may more or less fit a number of different antigen binding sites, a considerable array of different antibodies is likely to be produced, directed toward the several antigenic determinants of the antigen molecule.

COMPLEMENT. An enzymatic system of 11 serum proteins, 9 components, which act in a specific sequence, or cascade. The complement system is activated by many antigen-antibody reactions and is essential for antibody-mediated immune hemolysis and bacteriolysis. Complement participates in other biological reactions such as phagocytosis, opsonization, chemotaxis, and immune cytolysis (Park and Good 1974, Herbert and Wilkinson 1971).

The complement proteins have been named according to the numerical order of their role in the immune reaction, except for C4, which was named before the sequence was described (Koller 1981).

CONCANAVALIN A. A mitogen derived from *Canavalis ensiformis*, selective to immature T-cells (Sharma 1981e).

CONTACT DERMATITIS. Delayed hypersensitivity reaction of the skin resulting from exposure to chemical allergens, including environmental and industrial chemicals, drugs and cosmetics, and the catechols of poison oak and poison ivy (Ligo and James 1974, as reported in Gigli 1982).

CYTOTOXIC. Capable of producing cell lysis (Sharma 1981e).

CYTOTOXIC FACTOR. A lymphokine, released by a sensitized lymphocyte upon contact with antigen, that is capable of producing cell lysis (Sharma 1981c).

DELAYED HYPERSENSITIVITY. The state mediated by lymphocytes, which is evident only when lesions appear about 24 h after contact of the primed subject with antigen. This reaction is a manifestation of cell-mediated immunity and can be transferred to another animal with cells (Herbert and Wilkinson 1971).

DIRECT PFC. Plaque-forming cells producing antibody, primarily IgM, that can fix complement and lyse cells directly (Jerne and Nordin 1963).

EFFECTOR T-CELLS. Cells that, in delayed-type hypersensitivity, produce biologically active products called lymphokines, following contact with specific antigen (Luster et al. 1982b).

FREUND'S COMPLETE ADJUVANT (FCA). A water-in-oil emulsion adjuvant in which killed, dried mycobacteria (usually *M. tuberculosis*) are suspended in the oil phase (Herbert and Wilkinson 1971).

GRAFT-VS-HOST REACTION. The reaction of a graft containing immunologically competent cells against the tissues of a genetically different host (Herbert and Wilkinson 1971).

HAPTEN. A compound of low molecular weight, not antigenic itself, that can be combined with a protein carrier to form a product having antigenic properties and can thus elicit antibody formation (Sharma 1981c).

HELPER T-CELLS. T-lymphocytes that, through the release of soluble factors, activate B-cells and amplify their responses against many antigens (Sharma 1981b, Koller 1981).

HEMAGGLUTINATION. The clumping of a cell suspension by specific antibody (Gell and Coombs 1975).

HEMOLYSIS. The bursting of red blood cells. In immunology, this occurs when complement is added to the hemagglutinin reaction (Vos 1977).

HISTOCOMPATIBILITY ANTIGEN. An isoantigen carried on the surface of nucleated cells of many tissues which may induce an immune response that causes the rejection of a graft from one individual to another of the same species whose tissues do not carry that antigen (Herbert and Wilkinson 1971).

HISTOCOMPATIBILITY LOCUS. The locus on a chromosome at which the genes that determine the histocompatibility antigens are located (Herbert and Wilkinson 1971).

HUMORAL IMMUNE RESPONSE. The type of specific immunity that operates through antibody-producing cells and is transferable by serum (Vos 1977, Faith et al. 1980). The antibodies (immunoglobulins) are present in the plasma, lymph, and tissue fluids of the body and may become attached to cells (Herbert and Winkinson 1971).

Upon antigenic stimulation, a small number of B-lymphocytes, specific for that antigen, proliferate and differentiate into plasma cells that secrete antibody in a primary response or serve as memory cells for the secondary response (Faith et al. 1980).

HYPERSENSITIVITY. State of abnormal susceptibility or sensitivity, usually in reference to allergy (Sharma 1981b).

IMMEDIATE HYPERSENSITIVITY. Antibody-mediated immune reaction that results from the release of histamine and other vasoactive substances that can be transferred from one individual to another by serum alone. The antibody is usually of the IgE type, which fixes particularly to mast cells. The reaction usually appears within a few seconds to 30 minutes after contact with antigen (Herbert and Wilkinson 1971).

IMMUNE RESPONSE. The manifestation of the interaction of the organs and cells of the immune system, which is expressed through protective mechanisms such as rejection of allografts or delayed hypersensitivity (cellular immunity) or production of antibodies (humoral immunity). The responses of organisms to antigens are of two types: innate (or nonspecific) immunity and acquired (or specific) immunity (De Bruin 1976).

IMMUNE SUPPRESSION. Suppression of the immune response (Sharma 1981c).

IMMUNE SYSTEM. The organization of organs and cells which interact at various levels to effect the responses of the body to foreign entities (or antigens) (Miller 1975).

IMMUNIZATION. Administration of antigen to produce an immune response to that antigen. Administration of either antigen to produce active immunity or antibody to produce passive immunity to protect against the harmful effects of antigenic substances or organisms (Herbert and Wilkinson 1971).

IMMUNOASSAY. Analytical techniques that utilize antigen-antibody type reactions (Sharma 1981b).

IMMUNOCOMPETENCE. The capacity of the body to cope with infection and malignant growths. Immunocompetence has been characterized as (1) the establishment of barriers to maintain integrity and protect the body from the environment; (2) the capacity to recognize, neutralize, isolate, and reject foreign agents; (3) diverse reactions such as inflammation, granulomatous reactions, cell-mediated and humoral responses, and healing and repair processes; and (4) a process of "adoptive immunization" in which cells react in a greatly augmented or more effective manner to sequential exposures to a particular pathogen (Speirs and Speirs 1979).

IMMUNOGLOBULINS. Proteins, each of which is made up of two light and two heavy chains that are usually linked together by disulfide bonds. The various immunoglobulins differ from each other in primary structure even though some amino acid sequences are remarkably constant throughout phylogeny. All antibodies are immunoglobulins although all immunoglobulins may not be antibodies. Antibodies are produced in the body by plasma cells in response to stimulation by antigen, and they are capable of specific combination with antigen. Five major classes of antibody proteins, or immunoglobulins, have been identified in man: IgG, IgA, IgM, IgD, and IgE (Herbert and Wilkinson 1971).

The heavy and light chains of the immunoglobulins are symmetrically arranged, each consisting of a variable and a constant region. According to Goldstein et al. (1974):

> In the constant region of a given immunoglobulin class, the polypeptide chain is the same (or nearly the same) in its amino acid sequence. This similarity is common among various species and is found even in primitive organisms. In contrast, the variable region is composed of a variety of different amino acid sequences, while the total number of amino acid residues remains the same. The antigen binding sites are apparently located in the variable portions of the light and heavy chain. Generally, lymphoid cells differentiate in such a way that a given cell produces only a single immunoglobulin while all lymphoid cells together produce thousands of different immunoglobulins.

IMMUNOLOGICAL TOLERANCE. An acquired state of specific unresponsiveness to an antigen (Moreno 1982).

IMMUNOSUPPRESSION. Inhibition of the stimulation of an immune response by an antigen by a physical, chemical, or biological agent (Herbert and Wilkinson 1971).

INDIRECT PFC. Plaque-forming cells that produce antibody that either is insufficient for or is incapable of fixing complement unless it is complexed with antiglobulin antibody. Revealed in the PFC assay when developing serum is added to the plate (Dresser 1978).

INTERFERON. A protein (lymphokine) released by cells in response to virus infection. When taken up by other cells, interferon inhibits the replication of viruses within them. Interferon is also released after the injection of bacterial endotoxin (Park and Good 1974, Herbert and Wilkinson 1971, Sharma 1981a).

IgA. The major immunoglobulin component of secretions in the respiratory and gastrointestinal tracts and the second most abundant immunoglobulin class (10% of total) (Park and Good 1974, Rowe 1975), but present in relatively low concentrations in the serum. The molecule is synthesized locally in plasma cells of the submucosa; the secretory component is synthesized in the epithelial cells (Koller 1981). The IgA molecules of external secretions are primarily 11S, while those of the serum are mainly 7S. Although IgA does not fix complement, it may activate C3 by alternate pathways. IgA is active against viral and bacterial antigens at the sites of secretion, but the role of the antibodies in protection is not clear. It has been suggested that IgA antibodies may activate the alternate complement pathway and that they may be active in promoting phagocytosis and intracellular killing of organisms by peritoneal macrophages.

IgD. Prominent surface-bound immunoglobulin in the newborn; constitutes only 0 to 0.2% of immunoglobulins of normal serum (Koller 1981). Half-life about 3 days. Antibody activity against penicillin, insulin, milk proteins, diphtheria toxoid, and nuclear and thyroid antigens has been demonstrated in IgD molecules (Park and Good 1974).

IgE. The immunoglubulin that is associated with the reaginic antibodies active in Type I (see Section 3.4) allergic reactions (Bennich and Johansson 1971, Ishizaka and Ishizaka 1971). IgE is produced by the plasma cells of the regional lymph nodes and mucous membranes of the respiratory and gastrointestinal tracts (Koller 1981). The concentration of IgE in the serum is very small. It binds to tissue mast cells and blood basophils, and exposure of these cells to a specific allergen (the antigen to which the IgE is directed) leads to the release of histamine and other mediators. No protective role has been demonstrated for IgE antibodies. Rowe (1975) postulates that IgE antibodies may assist in protection against certain parasites either directly through pharmacological mediators or by the induction of an increase of vascular permeability allowing freer access of antibodies and cells from the blood.

IgG. The most abundant (85% of total) immunoglobulin in man, synthesized primarily during the secondary immune response. Has half-life of about 23 days (Park and Good 1974, Rowe 1975, Faith et al. 1980). It diffuses readily into extravascular tissue, where it can react with bacteria, parasites, and viruses (Park and Good 1974), and is responsible for long-lasting protection to these agents (Koller 1981). IgG is selectively transported across the placenta, and at birth is the predominant immunoglobulin in the circulation (Park and Good 1974).

IgM. Often the first immunoglobulin to be detected following antigenic challenge and the most effective specific first line of defense (Rowe 1975, Faith et al. 1980). The IgM antibodies constitute 5 to 10% of the total immunoglobulins in the blood (Park and Good 1974). One of the characteristic properties of IgM is its high molecular weight (900,000). It has a serum half-life 5 to 6 days.

KILLER LYMPHOCYTES. Special cytotoxic T-lymphocytes that participate in the delayed hypersensitivity reactions (Sharma 1981a).

KININS. Small polylpeptides that are released from a plasma alphaglobulin, kininogen, by proteolytic enzymes such as kallikrein. These enzymes are released when polymorophonuclear cells phagocytize immune complexes. Kinins are active as vasodilators and facilitate the increase of vascular permeability. They also cause contraction or relaxation of smooth muscle (Park and Good 1974, Herbert and Wilkinson 1971, Sharma 1981a).

LIPOPOLYSACCHARIDES. Compounds in which lipid is linked to polysaccharide. A common component of cell membranes. In immunology usually refers to components of O-antigen (endotoxin) complex of gram-negative bacilli such as *E. coli, Salmonella*, and *Bordetalla pertussis. E. coli* lipopolysaccharide is a thymus-independent antigen (Luster et al. 1978, Herbert and Wilkinson 1971).

LYMPH NODE. A kidney-shaped accumulation of reticuloendothelial cells and lymphoid tissue, connected to other lymph nodes by lymphatic vessels. These vessels enter the node at various places but leave it only at the hilus (a slight indention on the side of the node) on their way to the thoracic or right lymphatic ducts. The lymph nodes act as filters through which foreign materials must pass. These materials thus must come in contact with macrophages and lymphocytes; the lymph nodes are important centers for phagocytosis and for the initiation and development of the cellular and humoral immune responses.

The lymph node is divided into the outer cortical and inner medullary parts. The cortex consists primarily of dense lymphatic tissue which continues into the medulla as medullary cords. In the mid-cortex are thymus-dependent areas, which appear selectively depleted of cells in neonatally thymectomized animals. The cortex contains lymphatic nodules about 1 mm in diameter, which develop and disappear in response to various stimuli. These nodules contain dendritic macrophages which are capable of fixing antigens at their surface. In the embryo and the immediately postnatal stage they lack the central "germinal" areas which are thought to develop in response to antigenic stimulation. In old age the germinal areas may disappear. The medulla consists of the same cellular constituents as the cortex, but the medullary cords rarely contain nodules (Bloom and Fawcett 1969, Park and Good 1974, Herbert and Wilkinson 1971).

LYMPHOCYTES. During embryological development, lymphocyte progenitor cells migrate in the prenatal yolk sac through the liver to the bone marrow, with lymphocyte maturation occurring outside the bone marrow (Faith et al. 1980). Some cells enter the thymus, where they proliferate and mature under the influence of thymic epithelial cells. A small number of lymphocytes (T-cells) are released from the thymus, circulate through the body, and rest temporarily in thymus-dependent areas of the spleen and lymph nodes. Lymphocytes play a central role in both cellular and humoral immunity, but these lymphocytes consist of two distinct populations, T-cells and B-cells, with different functions and properties (Vos 1977).

LYMPHOCYTE TRANSFORMATION. The change in lymphocyte morphology when the cells are cultured in the presence of phytohaemagglutinin (or other mitogen) or of an antigen to which they are primed. The changes include: increased size, increased cytoplasm, and increased visibility of the nucleoli. After about 72 h the altered cells resemble blast cells (Herbert and Wilkinson 1971).

LYMPHOID ELEMENTS. Cells of the circulating blood that originate in the lymphatic tissue (e.g., lymphoctes and probably monocytes) (Bloom and Fawcett 1969).

LYMPHOKINES. Soluble nonantibody products of lymphocyte activation by antigen and mitogens that are thought to act as molecular mediators of cellular immune responses (Morely et al. 1978). Lymphokines exhibit the following characteristics: (1) produce increased vascular permeability following intradermal injection; (2) increase tritiated thymidine uptake by lymphocytes in culture; and (3) inhibit macrophage migration in vitro. Lymphokines retain biological activity after removal of the antigen that stimulated them. They may also be involved in both expression and regulation of lymphoid cell activity, and they may represent a pathway for the expression of T-cell function. Some lymphokines, such as the migration inhibition factor (MIF), the chemotactic factor (CF), and the macrophage-activating factor (MAF), influence macrophage functions (Faith et al. 1980).

LYMPHOPROLIFERATIVE RESPONSE. Lymphocyte proliferation induced by mitogens; the basis for in vitro assays of T- and B-cell function (Luster et al. 1982a).

LYSOZYMES. Enzymes found in many body fluids which lyse certain bacteria, primarily gram-positive cocci, and enhance the bactericidal action of antibody and complement for gram-negative bacteria (Park and Good 1974, Herbert and Wilkinson 1971).

MACROPHAGE. Nucleated cells that have been classified as part of the mononuclear phagocyte system, which replaces the concept of the reticuloendothelial system (Vos 1977). Macrophages, both fixed and free, are involved in and seem to be necessary for the initiation and the effector phases of humoral and cell-mediated immune reactions (Faith et al. 1980). The mechanisms of these activities are not clear. Macrophages do not clonally divide, as do lymphocytes, and they are not antigen specific (Pierce and Kapps 1976, as reported in Faith et al. 1980). They do, however, process the antigen, either breaking it down internally or retaining some material on the cell surface. It is this surface-bound antigen and perhaps a soluble factor from the macrophage which interact with lymphocytes in immune induction. Interactions of macrophages and sensitized lymphocytes in the effector phase of the cell-mediated immune response are probably mediated by lymphokines (Faith et al. 1980).

MACROPHAGE AGGREGATION FACTOR (MAF). Soluble material released by sensitized lymphocytes in culture (lymphokine), following exposure to antigen, that causes the aggregation of peritoneal exudate cells (Crowle 1975). MAF may be the same as MIF (Lolekha et al. 1970). Basis for in vitro test for delayed hypersensitivity.

MACROPHAGE MIGRATION INHIBITION FACTOR (MIF). Soluble material (lymphokine) elaborated by T-cells, and perhaps other cell types, following interaction with specific antigen in vitro. MIF inhibits the in vitro migration of normal exudate cells. (Bloom and Bennett 1966, Sharma 1981a). Can be used to test for delayed hypersensitivity in vitro.

MAF. Macrophage aggregation factor.

MEMORY CELLS. Long-lived small lymphocytes (B-cells), which are differentiated from virgin B-cells that have been stimulated by antigen. These cells are concentrated in the thoracic duct and are responsible for secondary antibody responses (Koller 1981).

MIF. Macrophage Migration Inhibition Factor.

MITOGENS. Molecules of certain lectins derived from plants or similar macromolecules that stimulate lymphocyte transformation (blast cell formation) in vitro. This is analogous to the in vivo formation of blast cells that precedes clonal division of B or T lymphocytes when there is an antigen challenge (Sharma 1981d).

MITOMYCIN C. Antibiotic obtained from *Streptomyces*. Has antitumor activity (Hawley 1981).

MYELOID ELEMENTS. Cells of the circulating blood that originate in the myeloid tissue (e.g., erythrocytes and granular leukocytes) (Bloom and Fawcett 1969).

NATURAL KILLER CELLS. A population of lymphocytes having natural cell-mediated cytotoxicity against various tumor cell lines (Luster et al. 1982b).

NONSPECIFIC IMMUNE RESPONSE. The means of disposal of foreign and potentially harmful macromolecules, microorganisms, or metazoa that does not involve the recognition of antigen or the induction of an immune response. Nonspecific immunity is generalized and includes the activities of phagocytic cells, microbial inhibitors (lysozymes), the interferon system, the complement system, and the kinin system (Herbert and Wilkinson 1971, Vos 1981).

NUDE MOUSE. Born without a thymus and unable to make T-lymphocytes. Responds poorly to thymus-dependent antigens, but mounts a good antibody response to thymus-independent antigens. Can serve as a laboratory model for a congenital immunodeficiency syndrome and may be useful in testing B-cell responses (Hayward 1982).

NULL CELLS. Lymphocytes that have characteristics of neither B- or T-cells. Useful in cellular cytoxicity assay (Luster et al. 1982b).

OPSONIN. (1) An antibody that, combined with antigen, facilitates the phagocytosis of that antigen by a macrophage or polymorphonuclear leucocyte. (2) Heat labile substances (nonantibody) found in blood plasma that facilitate phagocytosis. Probably activated components of complement, C3 in particular (Herbert and Wilkinson 1971).

PERIARTERIOLAR LYMPHOID SHEATH. T-lymphocyte-dependent lymphoid masses arranged around arteries in the white pulp of the spleen (Kociba 1981).

PEYER'S PATCHES. Gut-associated lymphoid tissue, histologically similar to secondary (peripheral) lymphoid tissue (Kociba 1981). T-cells of Peyer's patches are reported to have specific suppressor activity following oral antigen administration (Ngan and Kind 1978, as reported in Doe 1982).

PFC. Plaque-forming cells.

PHAGOCYTES. Cells, both fixed and free, that ingest and often digest large particles such as effete blood cells, bacteria, protozoa, and dead tissue cells. These include macrophages, Kupffer cells, and neutrophils. They are also active in ingesting foreign materials such as finely divided carbon (Park and Good 1974, Herbert and Wilkinson 1971).

PHYTOHEMAGGLUTININS. Lectins extracted from the beans of *Phaseolus vulgaris* or *P. communis* that stimulate lymphocyte transformation and causes agglutination of certain red blood cells (Herbert and Wilkinson 1971).

PLAQUE-FORMING CELLS (PFC). Cells obtained from the spleen or other lymphoid organs of an animal, immunized against SRBC or a protein antigen, which are mixed with sheep red blood cells (or antigen-coated SRBC if protein antigen was used) and molten agar, poured into a petri dish, and allowed to set. The dish is then incubated at 37°C. After incubation the release by each cell of hemolysins (antibodies capable of lysing red blood cells in the presence of complement) is revealed with the addition of complement to the dish. A clear zone of hemolysis appears around each antibody-forming cell. The PFC technique can be used to measure both primary and secondary response (Jerne and Nordin 1963).

PLASMA CELLS. Antibody-forming cells, differentiated from B-lymphocytes, that have a short half-life and secrete specific antibody (Sharma 1981b).

PPD. Purified protein derivative.

PRECIPITATION. The formation of a visible complex on mixing soluble antigen with antiserum specific for that antigen (Gell and Coombs 1975).

PRIMARY IMMUNE RESPONSE. The response of the body to the initial encounter with antigen. The response is weak, sluggish, and short-lived (Herbert and Wilkinson 1971).

PRIMARY LYMPHOID ORGANS. The thymus and bursa-equivalent (Luster et al. 1982b). The characteristics that distinguish primary organs from other lymphoreticular tissues are as follows: (1) the lack of cellular responses to antigen, such as those leading to antibody formation; most cell-mediated immune responses; and the induction of immunological memory (the medullary cells of the thymus, however, can respond to mitogens and participate in graft-vs-host reactions); (2) the intensive production of lymphoid cells in these organs, independent of antigenic stimulation; and (3) a reticular framework of epithelial cells that is not present in other lymphoreticular tissues (White 1975).

PRIMED. (1) Of a whole animal: activation of appropriate cells in lymphoid tissue by exposure to antigen so that further contact of a primed host with the same antigen usually results in a rapid and vigorous secondary immune response. (2) Of cells: activation of a given cell with antigen so that the cell can either produce more cells that have been so activated, or synthesize immunoglobulin, or mediate the reactions of cell-mediated immunity (Herbert and Wilkinson 1971).

PURIFIED PROTEIN DERIVATIVE (PPD). A soluble protein fraction, precipitated from the medium in which *M. tuberculosis* has been grown (Herbert and Wilkinson 1971).

RED PULP (SPLENIC). Consists of large numbers of blood-filled sinusoids in which phagocytosis of effete red cells takes place. Many phagocytes and plasma cells are found in the cords of Billroth, a modified lymphatic tissue that merges into the white pulp. In many mammals the red pulp of the spleen contains various sized groups of myelocytes, erythroblasts, megakaryocytes, and hemocytoblasts. The red pulp is also heavily populated with all the cells of the circulating blood (Bloom and Fawcett 1969, Herbert and Wilkinson 1971, Park and Good 1974).

SECONDARY IMMUNE RESPONSE. Antibody production after the second immunization. This response is characterized by faster development, higher levels, and greater persistence of antibody titers and a predominance of IgG (Koller 1981).

SECONDARY LYMPHOID ORGANS. The spleen, lymph nodes, circulting lymphocytes, GALT, and BALT (Luster et al. 1982b).

SPECIFIC IMMUNE RESPONSE. The result of the recognition of antigen in such a way that antibody or primed lymphocytes can react specifically with that antigen. Specific immunity includes cell-mediated immunity, humoral immunity, and immunologic tolerance (Herbert and Wilkinson 1971). A major characteristic of specific immunity is the establishment of immunological memory (Vos 1981).

SPLEEN. Situated in the bloodstream, unlike the lymphatic tissue, which is scattered in the lymph stream. The spleen, as does the lymph node, has a collagenous framework within which is suspended a reticular framework. There is also a capsule which is thickened at the hilus where the veins enter the organ and the arteries leave it. Continuations of the capsule, the trabeculae, penetrate the organ and form part of its framework. The reticular framework and the cells form the splenic tissue, which is composed of typical lymphatic tissue, the white pulp, and an atypical lymphatic tissue, the red pulp.

The spleen functions to (1) provide a site of differentiation for the lymphocytes and hematopoetic stem cells, (2) trap the blood-borne foreign and altered endogenous particles, (3) provide reservoir space for the circulating blood, and (4) form antibodies, particularly IgM, after stimulation by antigen in the circulating blood (Bloom and Fawcett 1969, Herbert and Wilkinson 1971, Park and Good 1974).

SRBC. Sheep red blood cells. Thymus-dependent antigen, commonly used in testing the humoral immune response (Vos 1981).

SUPPRESSOR T-CELLS. Lymphocytes that suppress antibody synthesis by inhibiting B-cells (Koller 1981).

T-LYMPHOCYTES. *T-cells* are stimulated by antigen to form a pool of sensitized, antigen-specific lymphocytes which then can function as memory cells, effector cells, killer cells, helper cells, or suppressor cells (Faith et al. 1980). T-cells do not produce antibody (Koller 1979a).

THYMUS-DEPENDENT ANTIGEN. Antigen, such as SRBC or tetanus toxoid, that elicit an immune response only when the thymus or thymus cells are present (Vos 1981).

THYMUS-INDEPENDENT ANTIGEN. Antigens, such as lipopolysaccharide, that can elicit an immune response by B-cells in the absence of the thymus (Moreno 1982).

THYMUS. In mammals the thymus is considered to be the primary lymphoid organ. In most mammals the thymus consists of two lobes located in the anterior part of the thorax, ventral to the trachea (Herbert and Wilkinson, 1971). Histologically, it consists mainly of lymphocytes distributed throughout distinct cortical and medullary areas on a network of reticular cells. These lymphocytes are continually being produced in the gland, but following the neonatal period only about 5% seem to leave. Hassal's corpuscles, which are groups of flattened, concentrically arranged cells, are present in the medulla.

During the neonatal period the thymus supplies small lymphocytes, those responsible for cell-mediated immunity, to the blood, lymph and thymus-dependent areas of the peripheral lymphoid organs.

The thymus also plays a part in humoral immunity. Neonatally thymectonized animals can mount only a poor antibody response to certain antigens such as SRBC and tetanus toxoid.

Investigations of thymic functions have established that the thymus is essential for the development of adaptive immunity. It appears to provide the microenvironment for the differentiation and proliferation of primitive stem cells migrating from the bone marrow; to elaborate a humoral factor that influences immunocompetency of lymphocytes elsewhere; and to foster cell-mediated immunity (Bloom and Fawcett 1969, Herbert and Wilkinson 1971, Park and Good 1974).

WHITE PULP (SPLENIC). Contains a stroma composed of reticular filters closely joined to primitive reticular cells and fixed macrophages. As in the lymph nodes, the meshes of the framework are filled with free lymphocytes of various sizes which form both diffuse and nodular lymphatic tissue. The amounts of dense and nodular lymphatic tissue vary in response to various stimuli. The lymphatic nodule contains a corona or peripheral zone and a central germinal center that may be involved in the immune response (Bloom and Fawcett 1969, Herbert and Wilkinson 1971, Park and Good 1974).

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4. INHALATION TOXICOLOGY

Michael G. Ryon

4.1. INTRODUCTION

Inhalation toxicology is the branch of toxicology that deals with exposure by the inhalation or respiratory route and with the resulting effects (Campbell 1976). Inhalation toxicology exists as a special subset of toxicology because of the unique exposure situation of the lungs and the special technique and equipment needs of the inhalation toxicity test.

The lung is the organ with the greatest direct contact between the environmental air (and the contaminants therein) and its vital, functional elements as a direct unavoidable consequence of living (Casarett 1975). This contact of the lung with the air is even more significant because of the tremendous surface area and number of structures involved. There are several hundred million alevoli in the human lung, with an approximate surface area of 100 m^2 when distended (Hatch and Gross 1964, Cambridge 1973). These are serviced by 2000 km of lung capillaries that are separated by only 1 μm from the gas in the alevoli (Hatch and Gross 1964, USDHEW 1977). Due to this large surface area/blood contact, even the rate of intake of highly soluble compounds can be ten times greater for the inhalation route than for the oral route. Added to these structural considerations is the tremendous amount of air (10 m^3) processed each day by the lungs (Hatch and Gross 1964). Thus, the contact with a potential lung toxicant can be extremely hazardous. The concern about the impact of air pollution exposure on man (Stokinger 1953) and the tremendous death rate from lung cancer [in 1967 in the United States over 50,000 deaths or one every 10 minutes (Saffiotti 1969)] underscore the importance of inhalation toxicity studies.

Inhalation toxicity also receives special emphasis because of the unique equipment and techniques required for its evaluation. Because the lungs not only are responsible for maintaining the oxygen supply and the excretion of carbon dioxide, but also have a role in several body defense systems, inhalation toxicity studies must deal with the systemic as well as specific lung damage (Casarett 1975). Thus, there is no way that only one standard and accepted protocol can be used for all inhalation studies (MacFarland 1975). However, base guidelines can be and have been proposed to ensure that minimum requirements of toxicity testing are being met; these guidelines should still allow the flexibility and freedom to substitute more stringent or comprehensive procedures (Gross 1981a, USEPA 1982). Inhalation studies are faced with unique problems with regard to determining dosage parameters and designing exposure conditions. A special technology has been developed to duplicate man's environment for study in the laboratory, including exposure chamber designs, generating and monitoring equipment for the various types of test atmospheres, and procedures for the evaluation of effects (Drew and Laskin 1973). These special requirements increase the average cost of a toxicity study using inhalation exposure to two or three times that of a study done by oral exposure (WHO 1978).

It is for these reasons that inhalation toxicology studies have received special emphasis. This report will discuss further the special needs of the inhalation study and in particular will cover basic equipment considerations and unique additions to test designs. Current guidelines for inhalation testing are not emphasized in this report; for a discussion of these, see Gross (1981a) and USEPA (1982).

4.2 GENERAL TEST DESIGN

4.2.1 Introduction

Toxicity tests designed to evaluate the effects of a chemical after inhalation exposure involve many of the same variables as tests using other exposure routes. All toxicity tests require the selection of test species, test duration, exposure regimen, dosage levels, and procedures to evaluate toxic effects. These variables will be discussed briefly in this section with emphasis on those aspects restricted to inhalation exposure.

4.2.2 Test Species

In selecting the most suitable species for inhalation tests, the anatomical and physiological similarity between human and test animal respiratory systems must be considered along with metabolic similarity and economic/practicality restrictions. The anatomical and physiological differences include variations in the structure of nasal cavities, trachea, and lower respiratory tree, in the site of particle deposition, and in the clearance and defense mechanisms (Giovacchini 1972). Table 4.1 lists some of the physiological variations between man and various test species. Because no single animal species is anatomically similar to man in all respects, researchers have used mice, monkeys, rats, rabbits, hamsters, guinea pigs, cats, and dogs for inhalation studies (Drew and Laskin 1973, WHO 1978).

McLaughlin, Tyler, and Canada studied the respiratory system of various animals, examining such subgross anatomical variables as the lobularity, pleura and interlobular septa, arterial supply to the pleura, and terminal bronchioles. They classified species according to their similarity to humans and concluded that the horse is anatomically the most similar to man (McLaughlin et al. 1961a, 1961b). The rat, rabbit, guinea pig, dog, cat, and monkey were considered to have basically the same type of lung structure and degree of relative similarity to man (McLaughlin et al. 1966). Among these species, Roe (1968) found that the smaller species

Table 4.1. Some Physiological Indices of Man and Animals[a]

Physiological index	Man	Dog	Cat	Rabbit	Guinea pig	Rat	Mouse
Body surface (m^2)	1.8	0.528	0.2	0.18	0.040	0.030	0.006
Relation body surface to body weight (m^2/kg)	0.0257	0.044	0.066	0.072	0.12	0.15	0.3
Basal metabolism (kJ/kg)	105	222	NG[b]	188	360	615	711
Frequency of respiration (min)	14-18	10-30	20-30	50-100	80-135	110-135	140-210
Size of alveoli (μm)	150	100	100	NG	NG	50	30
Surface of lungs (m^2)	50	100	7.2	5.21	1.47	0.56	0.12
Relation of lung surface to body weight (m^2/kg)	0.7	8.3	2.8	2.5	3.2	3.3	5.4
Inhaled air (mL)	616	40-60	NG	NG	1.75	0.865	0.154
Lung ventilation (mL/min)	8732	NG	1000	600	155	73	25
Relation of lung ventilation to body weight (mL/min/g)	0.13	NG	0.30	0.29	0.33	0.05	1.24
Consumption of oxygen (mL/kg/h)	203.1	3600	9420	522.7	2180	2199	3910
Elimination of CO_2 (mL/kg/h)	168.8	NG	NG	NG	NG	2650	4240
Coefficient of respiration	0.82	NG	NG	0.83	NG	0.82	0.85-1.33
Pulse frequency for 1 min	70-72	90-120	120-180	150-240	206-280	300-500	520-780

[a] Adapted from Sanockij (1970a) as reported in WHO (1978).

[b] NG = not given.

were markedly different from man, especially in the factors that affect particle deposition. The mouse, hamster, and rabbit lack the extensive tracheal or bronchial mucus-secreting glands that humans possess, while the rat possesses such glands only in the trachea. Although the cat does have these glands, its pattern of secretion is so unlike man as to make its use generally unsuitable. The guinea pig, which also has these glands, is limited in its applicability because of a predisposition to asthmatic-type attacks (Roe 1968). Roe felt that the dog and monkey were closer to man in gross anatomy and response but that their size reduced their applicability. Additionally, the monkey is useful only when laboratory-bred specimens are available. In contrast to some other researchers, Roe considered the mouse to be the preferred species for inhalation carcinogenesis studies due to its high susceptibility, which enhances the chances for tumor formation.

In an evaluation of dust retention in small animals, Palm et al. (1956) found that the retention pattern for the monkey was more similar to man than that of the guinea pig. In general, the smaller the animal the lower the rate of lung deposition for particles above 1 μm in diameter with less alveolar deposition. Also, the overall retention is greater in small animals, most likely due to greater impingement in the upper respiratory tract. However, Palm et al. (1956) did find that the most favorable particle size (1 μm) for alveolar deposition was similar for man and small animals. Another anatomical variable that influences particle deposition is the horizontal layout of respiratory tracts of quadrupeds, which is totally different from the vertical tract of man and other primates (Palm et al. 1956, WHO 1978). Carney (1979) found that, for evaluations of immunological sensitivity to particles, the rat was totally unsuitable due to sensitivity problems and that the guinea pig was a more suitable model. Brain and Mensah (1983) have reviewed many aspects of anatomical differences of species that can affect inhalation testing. They discussed variations in deposition, clearance, and biological response and concluded the following: (1) the fraction of aerosol actually deposited in the respiratory tract appears independent of body size; (2) different species breathing the same aerosols do not necessarily receive the same lung doses, and thus exposure concentration is not an adequate description of lung dose; (3) factors that affect the lung dose include changes in ventilation, collection efficiency, lung anatomy, and clearance mechanisms; and (4) interspecies variations in substance metabolism and innate biological responsiveness make it unlikely that lung damage will be the same even if the lung dose is equivalent.

The available literature clearly indicates that there is no species anatomically identical to man in all respiratory aspects. The most similar test species are generally the larger animals (horse, dog, and monkey), which are impractical for the majority of inhalation chambers and tests. It is therefore not surprising to find guidelines that recommend the rat (OECD 1979, Gross 1981a) or other rodent species, especially for acute tests

(Drew and Laskin 1973, NAS 1977). Other guidelines suggest that metabolic similarity should also be stressed, because systemic effects are important (Troy 1974), that a multiple species system is better (Hammond 1970, Drew and Laskin 1973), or that a rodent and nonrodent are needed for chronic studies (NAS 1977). A recent anatomical and physiological evaluation of the ferret indicates that its airways are more similar to humans than the dog's and other favorable aspects of its lung anatomy make it a more useful and less expensive choice for a nonrodent test species (Vinegar et al. 1981). As with other types of toxicity tests, no single species can be found to satisfy all the needs of inhalation tests, and the specific species chosen should be the one judged to be best suited for that particular chemical and exposure condition (Troy 1974).

4.2.3 Duration and Mode of Exposure

Similar to tests using other routes of exposure, there are three principal test durations for inhalation studies (acute, subchronic, and chronic). The initial evaluation of toxicity is often obtained through an acute test. This test is defined as a single uninterrupted exposure of high concentration, usually with a duration of less than 8 h (Drew and Laskin 1973, NAS 1977, WHO 1978). An acute test can provide various information, including approximate comparative toxicity of the chemical, the range of doses needed for studies of longer durations, and the nature of possible toxic effects (Drew and Laskin 1973, Troy 1974, NAS 1977). This information is provided primarily by determination of an LC_{50} value, which is the atmospheric concentration that will kill 50% of the test animals within a predetermined period of time (Drew and Laskin 1973, Carney 1979). Often the LC_{50} is a statistically derived value (OECD 1979) and is given in units of test substance weight per volume of air (mg/L, mg/m^3, or ppm) (Troy 1974, OECD 1979). Guidelines have suggested an exposure period of 1 to 4 h with a 14-day observation period (NAS 1977, Gross 1981a). The length of the observation period is critical, because a short period might miss delayed mortality and a long period could allow repair of any tissue damage (Carney 1979). EPA guidelines state that the observation period should be flexible; a length beyond 14 days could be justified by the toxic reactions, rate of onset, and length of recovery (USEPA 1982). Evaluations of acute toxic effects can include clinical observations (NAS 1977), airway irritancy responses (Giovacchini 1972, Cambridge 1973, Wells 1979), gross necropsy, and microscopic examination of the lungs or other target organs (NAS 1977, OECD 1979). Occasionally, no acute effects can be obtained even with the maximum concentration that can be generated (Carney 1979). In this case testing may be halted (OECD 1979) unless potential acute human exposure is expected (Carney 1979).

To initially evaluate toxicity resulting from repeated exposure to a substance, a subchronic test is usually performed. The duration of a subchronic inhalation test is no more than 10% of the lifespan of the test animal, with typical lengths of 14, 28, or 90 days (OECD 1979). An exposure time of 6 h/day is often used for these tests (Wells 1979, OECD 1979). Information on the nature of chronic toxic effects, detailed exposure-effect relationships, and range of doses for further chronic studies represents the principal results of a subchronic test (Drew and Laskin 1973). The degree of information obtained depends on the type of evaluations in the test design. For maximum information from the subchronic inhalation test, evaluations could include clinical observations, biochemical tests, hematology tests, gross and microscopic pathology, and pulmonary function tests (Giovacchini 1972, Carney 1979, OECD 1979, Page et al. 1980).

A chronic inhalation test is necessary to fully evaluate the effects of lifetime exposure to a substance. To simulate lifetime exposure in humans, the duration of the chronic test should be greater than 10% of the test animal's lifespan, which for most animals would be 1 to 5 years (NAS 1977, OECD 1979). Additionally, the exposure period should be patterned either after human occupational exposure (6 to 8 h/day, 5 days/week) or possible environmental exposure (22 to 24 h/day, 7 days/week) depending on the intended use of the product (Drew and Laskin 1973, WHO 1978). Procedures to evaluate the chronic effects could include clinical observations, biochemical tests, hematology tests, gross necropsy, microscopic pathology, and pulmonary function tests (NAS 1977, OECD 1979). To use these evaluations for the duration of a chronic test, large numbers of test animals (e.g., a minimum of 400 rodents) and exposure chambers are required (NAS 1977). Therefore the chronic test should be undertaken only after extensive preliminary testing has indicated the need for an evaluation of lifetime exposure effects.

As mentioned in discussion of chronic tests, exposure to the test substance can be scheduled on a continuous or an intermittent basis. Although this allows the substance to be evaluated in the potential mode of human exposure, there are certain factors of the two methods that must be considered.

With intermittent exposure (6 to 8 h/day, 5 days/week), the dose is pulsed with substantial periods of time, both daily and over the weekend, for recovery from the test substance. These periods of recovery can affect the toxicity of the chemical (Haun 1972, Drew and Laskin 1973, WHO 1978). Wright (1957) studied the effects of continuous versus intermittent exposure on dust retention in rats in a 3-month subchronic study and found that rats exposed for 2 h at a high concentration retained less dust (20 to 30%) than rats exposed for 20 h at a low concentration, even though both groups had the same daily concentration × time (C×T) dose. Sidorenko and Pinigin (1976) examined the two exposure modes in short-term studies with rats and mice and found that, generally, intermittent exposure is less harmful (in terms of mortality and time to onset of effects) than continuous exposure. As the intervals between exposures were increased in duration, the toxic effect of a substance in mice declined exponentially or proportionally, depending on the specific substance. In rats, the interrupted exposure had a smaller effect on the central nervous system and blood than did continuous exposure. However, Sidorenko and Pinigin did find some contradictory evidence in the rate of onset of effects with increasing time intervals between exposures. The varying response to discontinuous exposure is related to the quantity of test substance that can cross lung membranes and/or be absorbed by the aveolar blood supply during a given time and is perhaps related to the processes of adaptation and accumulation in the organism (Sidorenko and Pinigin 1976). Gardner et al. (1979) studied the variation between the two exposure modes using NO_2 and concluded that the concentration factor is more important than the length of exposure. When a constant C×T dose was employed, a short duration at a high concentration produced a greater effect than a long duration at a low concentration.

Intermittent exposure has some practical advantages that makes it easier and cheaper to use than continuous exposure. The chamber design can be much simpler, as there is no need to feed or water the animals during exposure (Drew and Laskin 1973, WHO 1978). Similarly, the atmosphere generating equipment and monitoring devices can be simpler because they operate only for 6 to 8 h/day (Drew and Laskin 1973, OECD 1979). The shorter period actually spent in the chamber also allows more animals to be exposed per cage (Drew and Laskin 1973). In large studies, these advantages can be substantial.

The advisability of initially evaluating a substance using actual exposure conditions has been questioned. The National Academy of Sciences (NAS 1977) has suggested that controlled studies can often give more informative data, especially if the actual use conditions are unique, and standard exposures also make comparisons of relative toxicity easier. Such tests basically evaluate the toxicity of the test substance (which is an inherent property of the compound) and do not fully address the hazard of the compound (which is a combination of toxicity and exposure conditions) (Gross 1981a). Additionally, even though intermittent exposure may simulate the time span of potential human exposure, actual concentrations in the environment vary, with peaks higher than the average concentration value. Thus, intermittent exposures may not be realistic or representative of the actual use conditions (Drew and Laskin 1973, WHO 1978).

4.2.4 Dosage

The design of inhalation exposure studies considers two factors of dosage: first, the appropriate number of levels (including controls) and, second, how to accurately determine the dosage.

The number of concentrations varies with the duration and goals of the test. Most guidelines recommend a minimum of three dose levels of the test substance for all studies (WHO 1978, OECD 1979, Page et al. 1980). Acute tests require that the dose levels be designed to produce a range of responses to permit construction of a mortality-concentration curve for calculation of an LC_{50} value. Subchronic and chronic tests should use a dose range with the high dose producing toxic effects, but not necessarily mortality, and the low dose producing minimal or no effects and being roughly equivalent to the No-Observable-Effect Level (NOEL) (WHO 1978, Gross 1981a). At least one intermediate dose is necessary if information on the dose-response relationship is desired (Page et al. 1980, Gross 1981a).

Incorporation of control groups is more extensive in chronic and subchronic tests. There should be at least one control group that undergoes the same procedures, except for actual exposure to the test substance, as the treatment groups. In addition, a control group is necessary to evaluate the administration vehicle, if one is employed. The use of a vehicle control group is also recommended for acute studies (OECD 1979).

The principal uncertainty associated with dosage in inhalation studies is the determination of the actual effective dose. For example, there may be variations in the dose to the lung, dose to the blood, and dose to the target organ. The factors that determine the dose delivered to the lung include, of course, length of exposure and atmospheric concentration of the test agent in the chamber (Roe 1968, MacFarland 1976). However, these factors describe, at best, only the amount of test agent available to the test animals. The critical value is the dose that reaches the sites of action in the lung (Hatch and Gross 1964, MacFarland 1975, Clark 1977). Many factors can affect this parameter, particularly test species variables such as minute volume of ventilation; frequency of respiration; bronchial dimensions, pulmonary and bronchial arterial bloodflow; rate of elimination including natural clearance mechanisms; metabolic conversion; activity levels of the animals; and percentage of retention (DuBois and Rogers 1968, Drew and Laskin 1973, NAS 1977, USDHEW 1977). Physical and chemical properties of the test agent also determine the effective dose, especially the solubility, diffusion coefficient, and, for aerosols, particle size (DuBois and Rogers 1968, NAS 1977). The degree that these factors affect the dose can also vary. It has been shown, at least theoretically, that the pulmonary factors may be less important in determining dose for tests of chronic duration than those of shorter exposures (DuBois and Rogers 1968). Further, defense mechanisms can concentrate, via phagocytic macrophages, a relatively diffuse and even exposure to create "hot spots" of high test agent concentration (USDHEW 1977). This effect may be especially important if thresholds for effects apply to the test agent.

In addition to these variables, there is often interference from other accidental routes of exposure. Clearance of the test agent from the lung via the mucociliary escalator can result in unintended gastrointestinal exposure (Clark 1977). Similarly, animals can ingest the test agent during grooming behavior or experience dermal exposure if the test is performed in a whole-body exposure chamber (Drew and Laskin 1973, Kawai and Nozaki 1978).

Because one of the primary objectives of the inhalation test is to elucidate upon the dose-response relationship, the variables and interferences discussed above have influenced how the inhalation toxicologist calculates dose and relates it to effects. The product of the atmospheric concentration (C) and the duration of exposure (T) is often used to determine dose-response relationships. This calculation does not produce a dose per se, as a dose by definition is a mass quantity (MacFarland 1976). However, it is still used to estimate the dose on the theory that the C×T dose is proportional to the inhaled dose, which is proportional to the absorbed or effective dose at the site of action (MacFarland 1976, Carney 1979). The principles behind this theory were revealed by Haber (1924) in his studies of chemical weapons and is often known as Haber's rule. This relationship holds true at most exposures, but at the high and low extremes the observed values deviate from the predicted values. Because the C×T dose is proportional to the real dose, it has been used to calculate $L(C \times T)_{50}$ data in acute tests and to develop dose-response data.

The main problem with the use of the C×T dose and Haber's rule to calculate dose-response relationships is that they represent a simplification of the real situation by ignoring interspecies and individual variations in minute volume and retention percentage (Weston and Karel 1946, Carney 1979). These factors are especially important because animal data are used as a basis for extrapolation to human exposure conditions. The large variation in the animal data could produce human exposure levels that are still within hazardous concentrations (MacFarland 1976).

Recognizing this potential problem, Weston and Karel (1946) developed a dose formula incorporating the minute volume (MV) and the percentage of inhaled agent that is retained (α). This Dosimetric Formula, dose = αCT•MV, can be considered a true dose because it results in a value with dimensions of a mass quantity. However, the Dosimetric Formula has proved to be difficult to use in practical situations. Values for the minute volume of animals at rest for different species are available in the literature, but because it is the values under the conditions of exposure that are important, studies should allow for measurement of the MV during actual exposure (MacFarland 1976). The retention percentage is even more difficult to quantify as the values vary depending on the chemical (particularly its solubility and particle size), duration of exposure, test species, and exposure chamber conditions (e.g., humidity). Despite these drawbacks, the Dosimetric Formula represents a better approach than does the C×T dose, especially for evaluating comparative toxicities or differences between routes of exposure (MacFarland 1976).

The difficulties associated with the C×T dose and the Dosimetric Formula have prompted researchers to experiment with alternative methods of evaluating inhalation doses, particularly in regard to the retained dose. Radioactive isotopes have been used to measure the actual amount of the test substance retained in the lung (Albert et al. 1967, Roe 1968). Griffis et al. (1981b) reported a method using acid-insoluble sodium to determine amounts of glass fiber retained in lungs after exposure to aerosols. Magnetometric techniques for measuring lung burdens of ferrimagnetic dust have also been reported (Oberdoerster and Freedman 1982). Circulating blood levels or rates of excretion have been used to estimate the body burden and are in some cases better estimates of dose than the C×T product (MacFarland 1976, Clark 1977). Such techniques still require additional development and validation before they can be incorporated into a routine inhalation protocol.

4.2.5 Observations for Effects

In inhalation studies, many methods have been used to observe and determine the toxic effects of the test agent. To detect systemic effects, most of the tests and observations are the same as those used in other toxicity studies (Poynter 1977). However, special procedures are used in observing toxic effects on the portal of entry, the lung. The observation of systemic effects will be only briefly discussed, with the majority of the discussion focusing on observations unique to inhalation studies. No attempt is made to suggest a rigid or standard protocol because the flexibility to choose between a battery of basic, simple tests and an array of special tests for inhalation effects can often be the most efficient and informative procedure (MacFarland 1975). With the vast array of special tests that are being developed and the many potential effects that could be monitored, the difficult task is to decide which aspects of inhalation toxicity to examine, as a truly comprehensive evaluation for all agents is impractical (Campbell 1976, Clark 1977).

4.2.5.1 Clinical observations

During and following exposure, observation of the test animals for clinical signs can often indicate systemic or lung damage. All animals should be observed at least daily and preferably twice a day (NAS 1977, OECD 1979, Gross 1981a). Observations should include any signs of irritation; changes in skin, fur, eyes, or mucous membranes; tremors; convulsions; salivation; bleeding; coughing; frothing at the mouth; presence and condition of urine and/or feces; lethargy; and changes in respiratory rate or pattern (NAS 1977, OECD 1979, Page et al. 1980). The observer should be particularly alert for moribund animals, in order to prevent loss from autolysis or cannibalism. In long-term studies, the animals can also be weighed once a week and the food consumption calculated.

4.2.5.2 Biochemical and hematological tests

Hematological and biochemical tests, including enzyme assays, should be performed on all animals before and after exposure (or at any interim sacrifice during exposure). Frequently performed hematology tests include hematocrit, hemoglobin concentration, erythrocyte count, total and differential leucocyte counts, prothrombin time, partial thromboplastin time, platelet count, and a blood smear examination (OECD 1979, Gross 1981a). Many biochemical tests have been used in inhalation studies, including alkaline phosphatase, serum glutamic-pyruvic transaminase, serum glutamic-oxaloacetic transaminase, blood urea nitrogen, total protein, albumin, globulin, cholesterol, fasting glucose, calcium, sodium, chloride, and potassium (OECD 1979). Such tests can give information on systemic effects or suggest additional special tests. The use of special biochemical evaluations of lung tissue has also been suggested, particularly for intermediate metabolism (Tierney 1974), and pulmonary surfactants (King 1974). The majority of biochemical tests indicating lung damage are currently in the developmental stage with many methods being modified from established hepatotoxicity evaluations (Witschi 1975). Although these studies have not yet identified a biochemical parameter that clearly indicates toxic effects in the lung, an encouraging beginning has been made (Witschi 1975). Biochemical analysis of pulmonary lavage fluid has also shown promise as an evaluative tool of inhalation toxicity. Henderson and coworkers (Henderson et al. 1978, 1979b, 1980) have used the lavage techniques developed by Mauderly (1977) as a short-term in vivo screen of acute toxicity in the rat and hamster. By measuring levels of various enzymes and components in the fluid (Table 4.2) after exposure to toxic agents, they determined that lavage was a more sensitive and earlier indicator of initial lung injury than was analysis of lung tissue. With continued development, it may be a useful technique for evaluation of chronic pathology. Other in vivo and in vitro assays may find some applications as alternatives to traditional inhalation toxicity studies (Brown and Poole 1983). These alternatives include in vitro studies using immunocompetent cells of the lungs, cytoxicity and cell morphology of cell cultures, and specific cellular biochemical activities as end points (Brown and Poole 1983).

4.2.5.3 Pathological observations

Evaluation of effects by pathology studies is the main method for determining inhalation toxicity. As in other types of toxicity tests, it is essential to have pathological observations that include both a thorough gross examination of all animals and a detailed microscopic examination of, at least, the high-dose, control, and lesion-bearing animals and the suspected target organs of animals at the intermediate levels (Gross 1981a).

The gross examination or necropsy is often more involved for inhalation studies than for other routes of exposure (Dungworth et al. 1976), with examination of additional respiratory tissues (nasopharynx, larynx, tracheobronchial tree, and parenchyma). The gross necropsy typically includes an examination of the external surfaces of the body; all orifices; and the cranial, abdominal, and thoracic cavities, including the organs and tissues contained within them (OECD 1979). Improved evaluations of the morphology of the nasal turbinates and mucosa have been recommended by Jersey and Kociba (1979), who suggest that grading the extent and

Table 4.2. Parameters Measured in Lavage Fluid

Parameter	Indication
Cytoplasmic enzymes: lactate dehydrogenase; glucose-6P-dehydrogenase organ specific enzymes; isoenzymes	Membrane damage
Lysosomal enzymes: acid phosphatase; beta-glucuronidase	Phagocytic activity
Membrane enzyme: alkaline phosphatase	Broken membranes
Mucin component: sialic acid	Increased mucin secretion
Cell count: total; differential	Inflammatory response

Source: Adapted from Henderson et al. (1980).

severity of the lesions can give dose-response relationships. During the necropsy, examination of the structures within the lung depends on the extent of gross damage; when edema or exudative lesions are absent, the airways and parenchyma are best examined after fixation (Dungworth et al. 1976).

After the necropsy, samples of all organs and tissues are preserved by a fixative solution. The lung requires special attention to ensure correct preservation. The preferred technique is to perfuse the lung via the airways, because this maintains the correct dimensions and configuration of the tissues while permiting rapid fixation (Dungworth et al. 1976, NAS 1977). The fixative (10% neutral buffered formalin or Karnovsky's formaldehyde/glutaraldehyde formula) should be perfused at a pressure of 25 to 30 cm of water for 1 to 4 h (Roe 1968, Dungworth et al. 1976).

The microscopic examination should include a wide array of body tissues and organs in addition to respiratory structures in order to assess both the systemic effects and the specific effects from inhalation. The exact organs to be sampled vary with the chemical and its potential effects; some suggested lists are given in Table 4.3. The assessment of specific lung

Table 4.3. Organs Suggested for Microscopic Examination[a]

NAS[b]	WHO[c]	OECD[d]
Bone	Accessory genital organs	Accessory genital organs
Bone marrow	Adrenals	Adrenals
Brain	Aorta	Aorta
Eye	Bone marrow, sternum	Brain
Gonads	Brain	Caecum
Heart	Caecum	Colon
Intestine, large	Colon	Duodenum
Intestine, small	Duodenum	Gall bladder (if present)
Kidneys	Gall bladder (if present)	Gonads
Liver	Gonads	Heart
Lung	Heart	Ileum
Lymph node, mesenteric	Ileum	Jejunum
Lymph node, peribronchial	Jejunum	Kidneys
Muscle, skeletal	Kidneys	Liver
Nasal cavity	Liver	Lung
Nerve, peripheral	Lung	Lymph nodes, axillary
Pancreas	Lymph nodes, axillary	Lymph nodes, mesenteric
Pituitary	Lymph nodes, mesenteric	Mammary glands
Prostrate	Mammary glands	Nerve, peripheral
Salivary gland	Muscle, thigh	Oesophagus
Skin	Nerve, peripheral	Pancreas
Spleen	Oesophagus	Pituitary
Stomach	Pancreas	Rectum
Thyroids	Pituitary	Salivary gland
Trachea	Rectum	Spleen
Urinary bladder	Salivary gland	Stomach
Uterus	Spinal cord (at three levels)	Thymus
	Spleen	Thyroid
	Stomach	Urinary bladder
	Thymus	
	Thyroid	
	Urinary bladder	

[a] Gross abnormalities or lesion-bearing tissues detected in gross necropsy should also be examined.

[b] NAS = National Academy of Sciences (1977).

[c] WHO = World Health Organization (1978, Chapter 5).

[d] OECD = Organization for Economic Cooperation and Development (1979).

effects requires sampling that is wide in distribution but specific in anatomic location because the size, diversity, and variable responses of the respiratory tissue are so great (Dungworth et al. 1976). Generally, samples to be taken include: (1) a longitudinal section of the distal trachea and the bifurcation into bronchi; (2) vertical sections of one lung and the cranial, middle, and caudal lobes of the other lung; (3) the proximal trachea; (4) the lobar bronchus; (5) the pharynx; (6) the larynx; and (7) the proximal and distal regions of the nasal sinuses and turbinates (Dungworth et al. 1976). These samples should be initially evaluated by light microscopy and/or scanning electron microscopy. If additional detail is desired, transmission electron microscopy can be used (Dungworth et al. 1976).

Occasionally, to fully elucidate the toxic effects, special pathology techniques may be employed, including autoradiography, histochemistry, and morphometry studies. Autoradiography is useful in determining cytokinetics of damaged pulmonary cells (Dungworth et al. 1976) and the correlation between deposition of particles and effect (Lisco 1959). Histochemistry is useful in localizing biochemical changes to specific cells or tissue types (Dungworth et al. 1976), whereas morphometic analysis is useful for accurately measuring the degree of damage and for statistically confirming subtle effects (Dungworth et al. 1976; WHO 1978). Although these procedures are not routine, they may serve a useful function in toxicity evaluations.

4.2.5.4 Respiratory function tests

The respiratory function tests are observation methods for assessing toxic effects that are uniquely used in inhalation studies. These tests allow the inhalation toxicologist to observe the effects of the test agent on breathing pattern, lung volumes, pulmonary pressures, flow volumes, and lung mechanics (Table 4.4). Other tests can be performed to determine changes in gas exchange and gas levels in the blood (Likens and Mauderly 1979). Generally, the respiratory function tests can be applied to intact animals, can be performed rapidly and repeatedly without affecting the animal, and will produce dose-response relationships, often with low variability (Swann et al. 1965, Alarie 1966, Hiett 1974, MacFarland 1976, WHO 1978, Wong and Alarie 1982, Juhos et al. 1985). One key to these methods is the understanding of baseline or mean values to allow interpretation of responses as toxic or nontoxic (Kennedy and Trochimowicz 1982). Because respiratory function can vary greatly between test animals, it is vital to have a series of baseline data for each animal prior to dosing. Concurrent evaluations of control animals can demonstrate any changes in respiratory function over time due to aging and repeated testing. The respiratory function tests are useful in acute studies for delineating effects and in chronic tests for following the development of effects (MacFarland 1976). They can also provide information on the mechanisms of action (Amdur 1958, MacFarland 1976), although such data are not always

Table 4.4. Respiratory Function Tests to Evaluate Breathing Patterns, Lung Volumes, Pulmonary Pressures, and Lung Mechanics

Test	Definition	Method of measurement[a]	Reference
Frequency (Respiratory rate)	Number of respirations per minute	Visual count; volume pneumograph (detects pressure changes in thorax); impedance pneumograph (uses electrical circuit to measure impedance change resulting from thorax movement); temperature change across nostrils; non-rebreathing valve	McCutcheon 1951, Alarie 1966, Barrow et al. 1971, Mauderly and Tesarek 1975, Crossland et al. 1977
Tidal volume (V_T)	Volume of air inspired during a quiet breath	Respirometer; plethysmograph used with a volume transducer; non-rebreathing valve (calculated value); pneumotachograph	McCutcheon 1951, Leong et al. 1964, Thomas and Morgan 1969, Diamond and Lipscomb 1970, Barrow et al. 1971, Barer et al. 1976
Minute volume (MV)	Volume of air expired per minute	Non-rebreathing valve; respirometer (calculated value); plethysmograph with volume transducer (calculated value); pneumotachograph	Truog and Standaert 1978, Leong et al. 1964, Diamond and Lipscomb 1970, Thomas and Morgan 1969
Vital capacity (VC)	Change in volume between maximal inspiration and maximal expiration	Plethysmograph (used with applied pressure); air injected into lung via syringe	Comroe et al. 1954, Drorbaugh 1960
Inspiratory capacity (IC)	Maximum volume that can be inhaled after a quiet expiration	Plethysmograph (used with applied pressure); air injected into lung via syringe	Comroe et al. 1954, Drorbaugh 1960
Expiratory reserve volume (ERV)	Volume that can be exhaled after a quiet inspiration	Plethysmograph (used with applied pressure); air injected into lung via syringe; pneumotachograph	Comroe et al. 1954, Drorbaugh 1960

Table 4.4 (continued)

Test	Definition	Method of measurement[a]	Reference
Total lung capacity (TLC)	Volume of gas contained in lungs at maximum inspiration	O_2 absorption technique (used with plethysmograph) calculated by adding residual volume and vital capacity values	Cavagna et al. 1967
Residual volume (RV)	Volume of gas remaining in the lung after maximal expiration	Calculated by subtracting expiratory residual volume from functional residual capacity values; determining amount of inhaled inert gas retained in lungs after exhalation	
Functional residual capacity (FRC)	Air remaining in lungs at end of quiet expiration	Inert gas dilution (uses tracheal cannula, O_2 chamber and percent N_2 to calculate value): $FRC = ERV + RV$	King 1966a, Caldwell and Fry 1969, Koo et al. 1976, Koen et al. 1977, Diamond and O'Donnell 1977
Intrapleural pressure	Pressure within the pleural space	Intrapleural catheter	Amdur and Mead 1958
Esophageal pressure	Pressure within the esophagus (approximates intrapleural pressure)	Fluid-filled or balloon-tipped esophageal catheter	Cavagna et al. 1967, Caldwell and Fry 1969, Koo et al. 1976
Transpulmonary pressure	Pressure difference between pleural pressure and tracheal opening	Differential pressure transducer connected to pleural or esophageal catheter and mask or breathing chamber	Amdur and Mead 1958, Palecek 1969

Table 4.4 (continued)

Test	Definition	Method of measurement[a]	Reference
Compliance	Distensibility of the lungs and thorax (relation of transpulmonary pressure to lung volume changes, $C = \Delta V/\Delta P$)	Dynamic: plethysmograph (for volume) and pleural or eosphageal pressure (calculated on analog computer, from oscilloscope displays, or by manual calculations); static: pressure volume curves generated by plethysmograph and air injected in lungs; forced oscillation of respiratory movements in a plethysmograph using volume changes measured by a pressure transducer	Davis and Morris 1953, Radford 1957, Amdur and Mead 1958, Dennis et al. 1969, Nattie 1977, Diamond et al. 1973, Hiett 1974, Decker et al. 1979
Resistance	Represents the frictional and viscous forces that occur with respiration (relation between transpulmonary pressure and $R = \Delta P/\Delta V$)	Dynamic: plethysmograph (for volume), pleural or esophageal pressure, and pneumotachograph (for flow) (calculated using same techniques as compliance); static: change in trans-pulmonary pressure at end expiration (using a pneumotachograph); forced oscillation of respiratory movements in plethysmograph	Amdur and Mead 1958, Mead 1960, Murphey and Ulrich 1964, Frank and Speizer 1965, King 1966b, Palecek 1969, Giles et al. 1971, Diamond et al. 1973
Forced vital capacity; forced expiration volume; maximum expiratory flow-volume	Dynamic forced respiratory characteristics useful for detecting changes in upper and lower airway resistance	Whole-body plethysmograph used with negative and positive pressure chamber; whole-body respirator and pneumotachograph	Diamond and O'Donnell 1977, Moorman et al., 1977

[a] Note: pneumotachograph can be used to measure all lung volumes except for RV. It is generally the most precise and easy to use means for measuring flow and volume (by intergration).

Source: Adapted from Likens and Mauderly (1979).

necessary in toxicological studies. One important advantage that respiratory function tests have over other evaluations of toxic effects is that they can quantify certain changes (e.g., reflex bronchoconstriction) at concentrations below the levels that produce morphological damage (Amdur 1958, WHO 1978). Therefore, they may allow measurement of such effects at levels of reversible rather than irreversible changes. Another advantage, particularly for chronic tests, is that the same animal's responses can be followed over the entire exposure period, thus avoiding the variability introduced by testing different groups at each point in time (Gross 1981b). Finally, similar data can often be generated for humans and test animals, which facilitates comparisons (WHO 1978).

The respiratory function tests do have disadvantages. First, they require special equipment and training for proper use (NAS 1977). Second, many of the tests (e.g., vital capacity or residual volume) used on humans cannot be used on test species without some modification or anesthesia because, as currently administered, the tests require the understanding and cooperation of the test subject (Roe 1968, MacFarland 1976). Third, some tests can be used only with anesthetized animals under highly unnatural physiological conditions (Roe 1968). Finally, the information gathered by these tests is rarely conclusive regarding the total effects of a test substance and must be supplemented by the other evaluation methods discussed in earlier sections, especially histopathology (Roe 1968, MacFarland 1976).

In addition to the tests of the mechanics, volumes, and pressures associated with respiratory function, tests to evaluate ventilation distribution, diffusion, and mucociliary action have been developed. A test that can be used to evaluate effects on the ventilation distribution is the nitrogen wash-out technique (MacFarland 1976). Generally, the technique measures the decline in nitrogen concentration of expired air after switching to pure oxygen inspiration. A rapid, exponential decline indicates normal ventilation distribution, but an uneven or less steep slope indicates an irregular ventilation distribution. Effects on the diffusion capabilities can be tested by determining the rate at which carbon monoxide or oxygen transfers from the alveolus to the hemoglobin (MacFarland 1976, Likens and Mauderly 1979). One way to test this process is a closed-circuit rebreathing technique using carbon monoxide. The rate of decline of the carbon monoxide indicates the diffusing capabilities. Diffusion capabilities can also be determined during a single vital capacity inspiration and expiration. Measurement of blood gas (oxygen, carbon dioxide) levels is another technique suggested for evaluating respiratory function. However, these tests can be difficult to perform in small animals and often require surgical approaches (Likens and Mauderly 1979). To determine effects on the mucociliary capacity of the lungs, a tracheal preparation can be taken from an exposed animal, test particles (e.g., pollen grains) applied, and their progress between two points observed. Another test uses radiolabeled particles in a live test animal and monitors their progress with a scintillation detector (Troy 1974). This latter approach was used by Kenoyer et al. (1982) to determine the toxic effects of ozone on short- and long-term clearance rates. They found that ozone exposure delays the initial clearance (perhaps by affecting ciliary beat rate or mucus changes), but the long-term clearance is accelerated (perhaps due to greater activity of deep-lung macrophages). These tests would be most useful in a study designed to evaluate only effects on the lung, because they are too complicated for use in a routine test of systemic toxicity.

4.3. EXPOSURE CHAMBER DESIGN

4.3.1 Introduction

The design of the exposure chamber should be one of the first factors considered in an inhalation test to ensure that the requirements of the exposure protocol are met. Specifically designed buildings and facilities are preferred for such tests. The building should have a high ceiling to accommodate chambers and necessary air ducts, have plenty of floor space to allow access to at least two and preferably all sides of the chamber, provide good illumination without allowing direct sunlight exposure of the chambers, and have an adequate air supply system with appropriate controls for humidity, temperature, and flow (Roe 1968, Drew and Laskin 1973, WHO 1978). The exposure chamber units should be designed to meet the majority of the basic requirements listed in Table 4.5. This chapter will deal with the necessary equipment to meet such requirements, including discussions of the various types of exposure systems, chamber design, air supply and exhaust systems, monitoring of the chamber, and use of specialized equipment. Consideration of test agent delivery and atmosphere generation equipment are covered in Section 4.4.

4.3.2 Types of Exposure Systems

This section will focus on the various operation modes possible for inhalation chambers. The administration of the test agent can be in a static or dynamic mode of operation. Also the animals can be exposed to the test agent via whole-body, nose-only or head-only exposure systems. The advantages, disadvantages, and uses of each system will be discussed.

4.3.2.1 Static systems

A static exposure system consists of a closed chamber into which a predetermined dose of the test agent is introduced and mixed (Lodge 1968). The test animals remain in this closed system for the duration of the test without any replacement of the test atmosphere. Obviously the lack of replacement air will limit the duration of exposure, because it allows depletion of the oxygen concentration, increased carbon dioxide levels, increased temperature (from buildup of animal body heat), and

Table 4.5. Inhalation Exposure Apparatus: Basic Requirements

Exposure chamber
Adequate size for exposure of sufficient animals for statistical evaluation
Even distribution of test gases, aerosols, or particle clouds
Temperature control
Humidity control
Easily cleaned and decontaminated
No hazard to personnel
Identical chambers available for untreated or vehicle-only treated controls
Test material
Gases — reliable control of concentration in chamber
Aerosols and particle clouds — reliable monodisperse system as well as control of concentration; vehicles, if used, should be without effect on respiratory system
Monitoring of exposure
Apparatus should be available for monitoring the concentration of gases, particle cloud density, and distribution of particle size at breathing zone, continuously during exposure
Safety
The absence of leaks in the chambers or connecting pipe systems should be ascertained repeatedly, preferably continuously, by a suitably designed monitoring system where hazardous agents are under test
The atmospheric pressure within the inhalation exposure system should be slightly below the ambient atmospheric pressure
Suitable traps and filters should guard both ends of all pipeflow systems

Source: Adapted from Hinners et al. (1966), as reported in Roe (1968).

increased humidity (MacFarland 1976, Clark 1977, NAS 1977). An exposure duration of 0.5 to 1 h has been suggested for smaller static systems (MacFarland 1976).

In addition to the problems associated with a finite volume, there is also a loss of test agent by deposition on the chamber walls or by removal for sampling purposes (Lodge 1968, Drew and Laskin 1973). Although many of these problems can be eliminated or reduced by use of a large-volume chamber (e.g., a minimum of 500 L/kg test animal weight for every hour of exposure, Clark 1977), nonintrusive sampling techniques, or nonreactive wall materials, there is still one major disadvantage. Because the total amount of test agent must be introduced at the beginning of the exposure, the concentration declines with time from an initial peak higher than the desired exposure to a final level below the desired exposure. Even though the average concentration value of the entire exposure may be at the desired level, the variations introduce uncertainty and prevent the uniform exposure concentration that is preferred for inhalation studies (MacFarland 1976).

Despite these drawbacks, there are several situations for which the static system is the best choice. Static systems are useful for assessing acute toxicity and in performing preliminary pilot studies (WHO 1978). Also, if the test agent is available only in limited quantities, a static exposure should be used (MacFarland 1976). A static system is likewise preferred in the testing of biological aerosols as these are difficult to generate continuously (Drew and Laskin 1973). The majority of inhalation tests, however, will use the dynamic system.

4.3.2.2 Dynamic systems

A dynamic exposure system consists of a chamber that has a continuous flow through of air and test agent at a constant rate and in a predetermined ratio (Silver 1946). The constant airflow avoids many of the problems that limit the use of static systems and is usually generated by placing an air pump in the exhaust line of the system. Such a placement creates a negative pressure in the chamber, which will aid in preventing loss of the test agent through leakage (Silver 1946, MacFarland 1976). Occasionally, to ensure an even airflow, buffer tanks are used to eliminate pump-induced flow fluctuations (Silver 1946). Usually the test animals are inserted in the chamber prior to exposure and then the gas flow is started (MacFarland 1976). This creates an exposure curve of the form shown in Figure 4.1. If immediate exposure to a constant concentration is desired, an airlock device, usually built into the chamber door, can be used (Silver 1946). The animals are placed in the airlock, the outer door is closed, and the airlock is opened to the chamber atmosphere. This produces the exposure curve shown in Figure 4.2.

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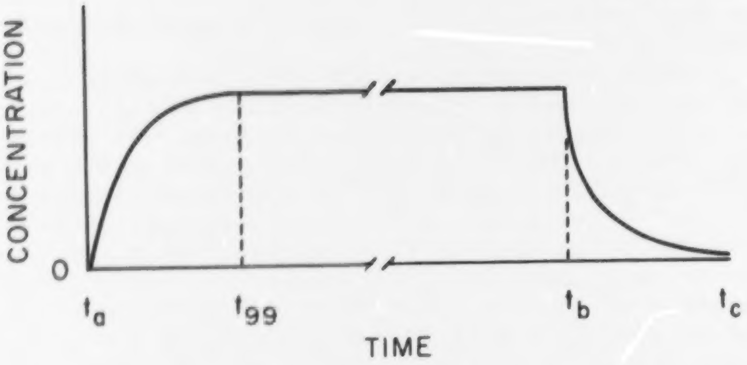

Figure 4.1. Concentration-time relationships in a chamber operated for a long period of time. t_a = Start of flow through chamber; t_{99} = time at which equilibrium concentration is reached; t_b = time at which test agent is no longer added to airflow; and t_c = end of airflow through chamber. (Source: Adapted from MacFarland 1976. Used with permission of Academic Press.)

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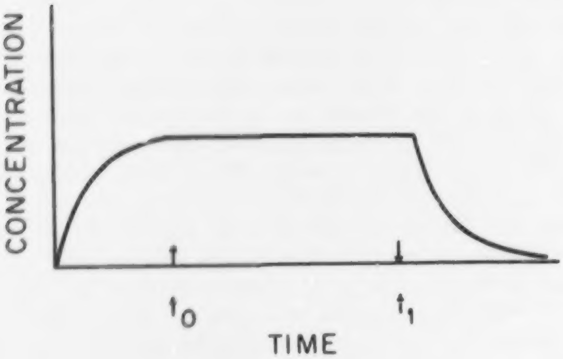

Figure 4.2. Time-concentration curve for exposure to constant concentration using air-lock mechanism. t_0 = Animals introduced into chamber and t_1 = animals removed from chamber. (Source: Adapted from MacFarland 1976. Used with permission of Academic Press.)

In normal use situations, it takes a certain amount of time for the chamber concentration to reach the desired level (see Figure 4.1). Silver (1946) developed the following equation to calculate this time lag:

$$C = \frac{w}{b}\left(1 - e^{\frac{-bt}{a}}\right)$$

where

C = the concentration (mg/L) at time t,
w = mg agent introduced per minute,
a = the volume of chamber (L),
b = the volume of air passing through the chamber each minute,
e = natural logarithm

Two assumptions, constant flow and complete mixing, which are generally satisfied, are required for this equation (MacFarland 1976). Because this is an exponential rate, Silver assumed a value of 99% to be sufficiently close to the desired equilibrium value for testing; based on that value the following equation can be used to determine when the chamber has reached equilibrium:

$$T_{99} = 4.6052\frac{a}{b} \ .$$

MacFarland (1981) points out that, after the T_{99} concentration level is reached, the aerosol distribution should be uniform within the chamber, as inlet air would not differ by more than 1% from the chamber concentrations in most chamber arrangements. Other numerical values for the constant (4.6052) are given by Silver (1946) for percentages lower than 99%. Because the T_{99} value depends on chamber size and flow rate, one could reduce the time to equilibrium by increasing the flow (for both test agent and total flow) above that required for the desired exposure (MacFarland 1976). Once the T_{99} equilibrium has been reached, the flow rate can be reduced. Another technique to avoid the delay would be to use dual rate supply systems. With the latter method, one must be careful that the concentration does not exceed the desired level (MacFarland 1976). The T_{99} equilibrium is a theoretical value, and, although the chamber should be uniformly mixed, the actual chamber concentrations may be lower due to losses of test agent from adsorption on walls or animal fur and uptake by the animals. Therefore, monitoring of the actual concentration in the chamber is always necessary to ensure proper exposure levels (Lodge 1968, Drew and Laskin 1973).

The appropriate flow rate to be used in dynamic chambers must fall within a limited range if the chamber is to avoid the heat buildup and oxygen loss problems that occur with static chambers. MacFarland (1976) discusses the ranges of flow rates commonly used and recommends the following equations:

F = 0.1 to 0.2V for gases,
F = 0.2 to 0.5V for volatile vapors,
F = 0.5 to 0.7V for light aerosols, and
F = 0.7 to 1.0V for heavy aerosols,

where

F = flow rate in liters per minute
V = chamber volume in liters.

For example, if the chamber size is 1000 L, the flow rate should be in the range of 100 to 200 L/min for a gas, but 700 to 1000 L/min for a heavy aerosol. If flow rates are calculated in this manner and test animals loaded in the chamber at a density (animal volume per chamber volume) of 5% or less, the use of special air conditioning systems are not necessary. EPA guidelines require a dynamic air flow of 12 to 15 air changes per hour to ensure an oxygen content of 19% (USEPA 1982). The flexibility shown by the dynamic exposure system makes it the preferred choice for chronic, subchronic, or any repeated exposure studies where test agent availability is not a limiting factor. Dynamic chambers are required for testing within the EPA guidelines (USEPA 1982).

4.3.2.3 Whole-body exposure systems

Whole-body exposure systems, especially when operated dynamically, are the most frequently used exposure systems in inhalation testing. The animals are placed in cages within the exposure chamber and exposed to the test agent atmosphere. Whole-body exposure requires no surgical or anesthetic preparation prior to testing (MacFarland 1976). The animals are free to move within their cages, and the use of exercise devices can provide information on the effects in active animals. With whole-body exposure systems, large numbers of animals or several different species can be exposed simultaneously in one chamber (MacFarland 1976). Uniform exposure conditions for all animals are obtained even when large numbers of animals are exposed. The whole-body system does, however, have some disadvantages. Deposition of the test agent (especially aerosols) on animal fur with the potential for ingestion during grooming behavior is a major problem (MacFarland 1976, Nettesheim and Griesemer 1978). The animals should be housed in individual cages to reduce such ingestion and to avoid dosage interference resulting from filtration through the fur of huddling animals (MacFarland 1976, Clark 1977). When whole-body exposure systems are operated in the usual dynamic mode, large quantities of the test agent are required, which can be a disadvantage (MacFarland 1976), and, if the test agent is extremely hazardous, special handling techniques and equipment, such as glove ports, must be used to protect the laboratory technicians (Nettesheim and Griesemer 1978). These drawbacks have stimulated the development of the alternative exposure systems discussed in Section 4.3.2.4.

4.3.2.4 Nose- and head-only exposure systems

In some inhalation studies, exposure of the whole-body is not acceptable; for these situations nose- or head-only exposure systems have been developed. Basically, these systems consist of a central chamber into which the test agent is introduced and separate holding devices that position the animal so that only the nose or head is protruding into the test atmosphere. The design of the central chamber may be circular, hexagonal, or linear and be constructed of Plexiglass® or stainless steel (Thomas and Lie 1963, MacFarland 1976). The nose, muzzle, or neck of the exposed animals must be sealed to prevent loss or dilution of the test agent. The holding devices include cylindrical Plexiglass® containers (e.g., plethysmograph) used mainly for rodents, sling arrangements for dogs, and adjustable chairs for primates (MacFarland 1976). If exposure of many animals is desired, cylindrical or hexagonal chambers can be stacked to provide a larger central chamber for exposure (Drew and Laskin 1973). If a linear arrangement is used to repeatedly expose large numbers of animals, the positions of the animals must be rotated between exposures because the linear arrangement can affect test agent concentrations. An alternative method involves using a molded face mask to deliver the test atmosphere. This system also requires some type of restraining device and an airtight seal between mask and animal to prevent air loss and eliminate dead space. Latex molding techniques have proved satisfactory for maintaining a tight seal and have been fitted to dogs, rodents, and primates (Dubin and Morrison 1969, MacFarland 1976).

The nose- and head-only systems are most useful for studies where no or little dermal or oral exposure can be tolerated (Drew and Laskin 1973, MacFarland 1976). Both systems reduce the total area exposed, with the face mask system exposing the least surface area. A comparison of a whole-body exposure system and two nose-only exposure systems (a cylindrical polycarbonate tube and a stocklike restrainer) using radioactive tracers indicated that the nose-only exposures were much more accurate for delivery of inhalation doses (Henry et al. 1983). Data show that only 13% of the total dose was deposited in the lungs of mice during a 130-min whole-body exposure, with 60 to 80% of the dose eventually reaching the gastrointestinal tract. The nose-only exposure systems delivered approximately threefold to fivefold more test material to the lungs and threefold less material to the gastrointestinal tract under the same test conditions. These systems are also advantageous when the test agent is extremely hazardous (e.g., radioactive aerosols) or when it is available only in limited quanities (Drew and Laskin 1973, WHO 1978). For extremely hazardous substances, the entire exposure apparatus can be set up within an enclosed inhalation chamber with glove boxes or a rolling drawer (Moorman 1978). This provides a double measure of safety, enables animals to be manipulated, and allows rapid reestablishment of exposure conditions. Other advantages include the potential for slower air flow through the chamber, because body heat buildup is not a problem inside the chamber, and the capability for rapid exposure of the animals to the test atmosphere (especially for aerosols) due to the small chamber size (Wells 1979). One unique application of these systems is a design that exposes animals either dermally and/or by inhalation, depending on how the animal holder tubes are inserted into the chamber (Francovitch et al. 1982). Most applications of these systems are for acute testing, as such tests do not require feeding or watering of the animals during exposure (Drew and Laskin 1973). Long-term testing is difficult due to the need to restrain the animals.

One of the major disadvantages of these exposure systems is the difficulty of exposing large numbers of animals (MacFarland 1976). Often a large crew of technicians is needed if many animals are to be exposed (Nettesheim and Griesemer 1978). Other disadvantages are caused by the extra handling and restraints required. The physical stress created or the anesthetic required to avoid this stress can affect the outcome of the exposure (Drew and Laskin 1973). One aspect of physical stress is the buildup of body heat within the restraining tube. Perforations in the tube or other means of ventilation can offset this disadvantage (Kennedy and Trochimowicz 1982). Nettesheim and Griesemer (1978) found reduced body weight gain and increased adrenal gland weight in animals exposed by these systems. However, Smith et al. (1980) reported on a nose-exposure system using a polycarbonate tube for chronic aerosol exposures of fibers that did not affect stress as measured by body weight, temperature, blood counts, and plasma corticosterone levels. Thus, some of the nose- and head-only exposure systems may be useful for long-term, repeated exposure tests but are best utilized for the specialized exposure conditions described.

4.3.3 Chamber Design

As mentioned previously, the majority of inhalation chambers are designed for dynamic, whole-body exposures. The discussions in this section will pertain to this type of chamber unless otherwise specified. In designing an inhalation chamber the principal objectives are performance and ease of operation (Fraser et al. 1959). Performance refers to the ability of the chamber to meet the requirements of the particular inhalation study being undertaken. Ease of operation includes ready accessibility of animals for sampling and handling, and maintenance and sanitation considerations. Due to the wide variety of potential test substances and their different properties, many modifications of a few basic chamber designs have been developed. This section will deal with the main design considerations of shape, size, construction materials, and some operational features of the basic chambers. Modifications of these basic chambers will be discussed, depending on their potential utility.

4.3.3.1 Chamber shape

The shape of inhalation chambers is determined by requirements for uniformly distributing the test agent and minimizing the internal surface area in order to reduce adsorption and deposition of the test agent (Silver 1946). Theoretically, a large volume with a small surface area is the desired condition. This is achieved best by a chamber of spherical shape (Fraser et al. 1959); however, such a chamber is difficult to build and fit with doors or windows (MacFarland 1976). The practical solution is to use a chamber with a cubical shape, but this results in 25% more surface area than does the sphere (Frazer et al. 1959, Hinners et al. 1966, MacFarland 1976). An intermediate design is a hexagonally shaped chamber, but the gain in efficiency is usually not worth the increased cost of construction (MacFarland 1976). By adding pyramid (or cone) structures to the top and bottom with air entering at the top and exhausting at the bottom, and by avoiding animal placement in the corners, the air distribution efficiency can be increased and a cubical chamber used for most applications (Fraser et al. 1959, Roe 1968).

Large-scale exposure chambers, incorporating most of the shape considerations discussed above, are best represented by the Rochester and the New York University chambers. The Rochester chamber (Figure 4.3) is a hexagonal-shaped chamber with conical additions to the top and bottom (Fraser et al. 1959). The New York University chamber (Figure 4.4) is based on a cubical shape with pyramid additions (Drew and Laskin 1973) and is considered the system of choice for repeated exposure studies (NAS 1977).

Other large-scale designs have been used for specialized exposures or when unique equipment is available. Spherical chambers composed of clear Lucite® plastic have been used for exposures to carcinogens at low flow rates and can be built at a lower cost than steel chambers (Stuart et al. 1970). The Longley chamber is a modification of the standard rectangular design and is subdivided into two or four separate sections (MacEwen 1978). This allows simultaneous exposure of control and three dose levels of test animals. Also, the design is flexible enough to permit testing of multiple dose levels for acute studies.

Carcinogenic studies often require specialized chamber designs because of the long duration of the studies, the large number of animals, and need to isolate the carcinogens. Laskin et al. (1970) designed a system of three box chambers that are connected by internal sliding doors. One chamber is used for the inhalation exposure while the other two chambers house the control and experimental animals. Pass boxes and neoprene gloves built into the system allow the animals, food, and wastes to be introduced into, moved within, or removed from the system. Thus, the animals never have to be removed from the unit and can be totally isolated. Entire rooms have also been designed as large-scale exposure units for exposures of many animals. Chambers with an 2.4 m × 2.4 m floor area and a submarinelike door hatch at each end were designed at Dow Chemical Corporation for

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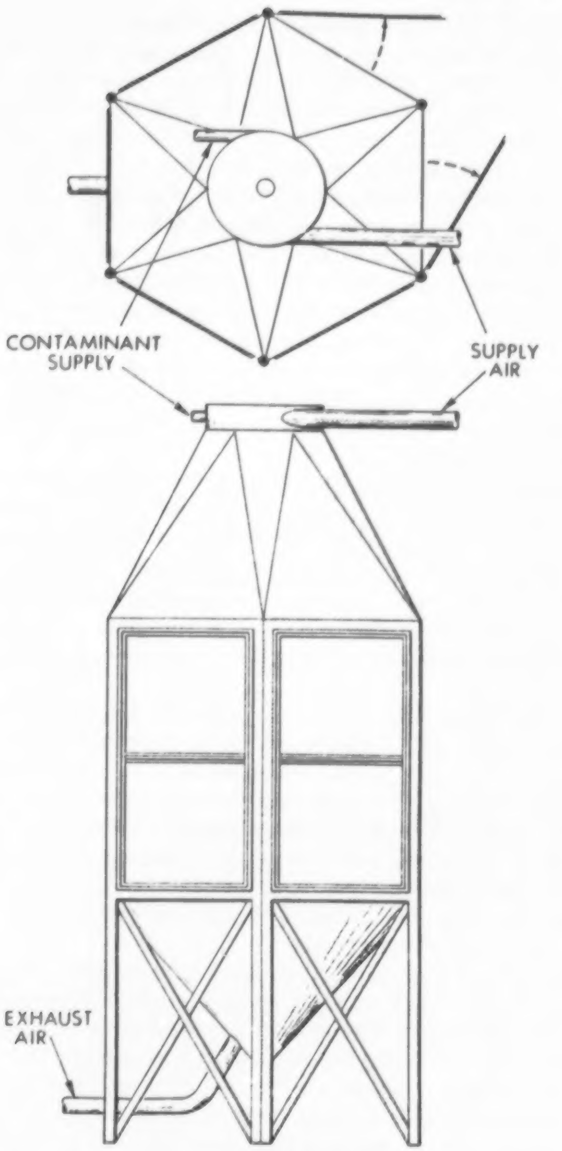
CONTAMINANT
SUPPLY
SUPPLY
AIR
EXHAUST
AIR

Figure 4.3. Schematic diagram of the Rochester exposure chamber. (Source: Adapted from Drew and Laskin 1973. Used with permission of Academic Press.)

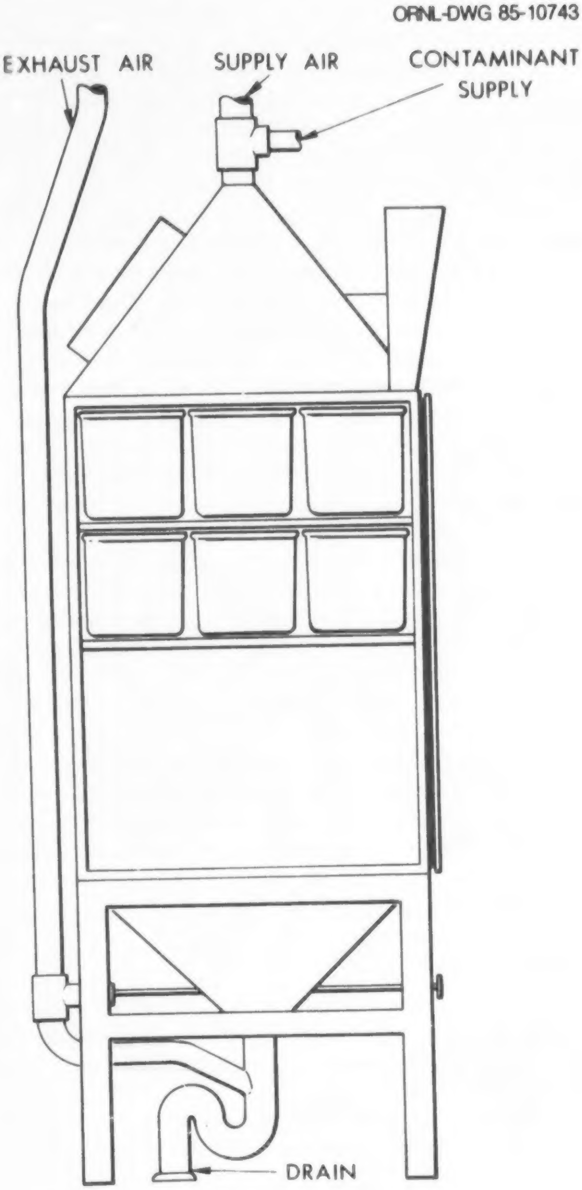
ORNL-DWG 85-10743
EXHAUST AIR
SUPPLY AIR
CONTAMINANT SUPPLY
DRAIN

Figure 4.4. Schematic diagram of the New York University exposure chamber. (Source: Adapted from Drew and Laskin 1973. Used with permission of Academic Press.)

exposure of large numbers of test animals (MacEwen 1978). The doors were connected to a clean corridor and a "dirty" corridor to control the spread of the test materials via the movement of animal racks in and out of the chamber. The current state of large chamber design is "one chamber–one room–one project," which achieves the performance of individual experiments in an integrated framework (MacEwen 1978).

Smaller-scale exposure chambers offer more variations in design than the larger units because they are used for single exposures or short test durations. One of the first and simplest small-scale designs (Figure 4.5) is a glass bell jar mounted and sealed to a wooden or metal base with ports for air inlet and exhaust (Fraser et al. 1959). These can be operated in a dynamic or static mode and are usually enclosed in a fume hood for added safety. Similar to this approach, Laskin and Drew (1970) designed a Lucite cylinder with injection molded domes at each end, supported on a wooden frame. The top dome can be removed for access and is sealed with rubber O-rings. This system is relatively inexpensive and its size (36 cm in diameter and 60 cm in length) makes it ideal for acute or pilot studies. Another approach to small-scale units is to scale down a larger design. Fraser et al. (1959) describe a dynamic, cubical unit with two stainless steel walls, one Plexiglass® wall, a removable Plexiglass® door, and a maximum size of 25 ft^3 (7.6 m^3). Drew and Laskin (1973) described another scaled-down unit, called a California Hood, that utilizes the New York University chamber design mounted within a square exhaust chamber. Besides these small-scale exposure chambers, a unique chamber design of a converted, cylindrical human respirator with a horizontal layout and a once-through airflow from end to end has been described for short-term exposure of monkeys (Thiede et al. 1974). An important factor that should be incorporated into any of the small chamber designs is adaptability of the components (Fraser et al. 1959). Because the purpose of these designs is to perform acute, pilot, or other limited studies, component adaptability will allow the unit to be used repeatedly for different purposes or test chemicals and consequently will reduce cost.

4.3.3.2 Chamber size

As discussed in the previous section, exposure chambers occur in a wide range of sizes. The most important consideration for size is the volume occupied by the animals in relation to the total chamber volume. The animal volume should never be greater than 5% of the total volume to prevent heat and carbon dioxide buildup and to avoid interfering with air circulation (Silver 1946, Fraser et al. 1959). In this regard, it is better to use a chamber that is too large rather than one that may be too small (MacFarland 1976). The size range of large-scale chambers that maintain good air distribution (especially for dusts and aerosols) is 1 to 8 m^3 (MacFarland 1976). Chambers of a size outside of this range can be used if alternative methods for mixing and distributing the test atmosphere are used or if a static system is employed. For example, Schreck et al. (1981)

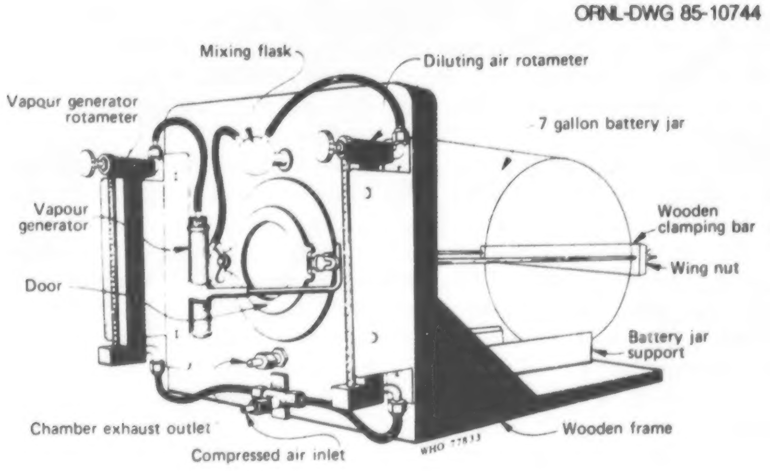
ORNL-DWG 85-10744
Mixing flask
Diluting air rotameter
Vapour generator rotameter
7 gallon battery jar
Vapour generator
Wooden clamping bar
Wing nut
Door
Battery jar support
Chamber exhaust outlet
Wooden frame
Compressed air inlet
WHO 77833

Figure 4.5. Small-scale exposure chamber based on a bell jar design. (Source: Adapted from Leach 1963, as reported in Drew and Laskin 1973. Used with permission of Academic Press.)

describe a large Rochester-type chamber (~12.6 m^3) that uses an exhaust manifold to aid in maintaining an acceptable air distribution. The large system was designed to reduce maintenance time and allow large numbers of test animals to be chronically exposed. However, it is generally recommended that the chamber size be within the 1-to-8-ft^3 range and that more chambers be used if space is insufficient for the test purposes.

4.3.3.3 Chamber materials and construction

Ideally, materials used in constructing the inhalation exposure chamber should be determined by the nature of the test agent (Fraser et al. 1959). However, most chambers will be used repeatedly for tests of different substances and they are generally constructed of materials that are highly resistant to a wide range of substances. The interior of the chamber should be simple in design and have smooth finished surfaces with no sharp edges.

The walls are usually constructed of, or lined with, a smooth nonabsorbent material (Fraser et al. 1959). Common wall materials include aluminum, stainless steel, safety glass, and plastic. If less resistant materials are used (or if the test agent reacts with one of the above materials), the walls can be lined with glass, enameled, or lacquered (Silver 1946). Galvanized iron riveted together has been suggested for the pyramid funnels, depending on the test substance (Timbrell et al. 1970). Stainless steel is the most commonly used metal, but it is susceptible to crevice corrosion after welding. If welding is used, Type 304 stainless steel is preferred due to its resistant properties (Hinners et al. 1968). In large chambers, it may be necessary to reinforce the walls with 3/4-in. pipe to provide support (Hinners et al. 1968).

The door and observation ports are made of plate glass or Plexiglass-type plastics (Fraser et al. 1959). The door should permit easy access and be as large as practically possible. If a large glass door is used, stainless steel framing and cross members may be necessary for support. The door can be of a sliding design (either vertically or horizontally) or hinged with full-length piano hinges (Fraser et al. 1959, Hinners et al. 1966). Toggle clamps, construction clamps, or magnetic devices have been used to maintain the tight seal for the long periods that is necessary for subchronic and chronic inhalation studies (Hinners et al. 1968, Timbrell et al. 1970). A stainless steel angle around the interior edge of the door will prevent the test animals from clawing or chewing the door gaskets (Hinners et al. 1968). Seals for the door and removable ports can be made of neoprene, PVC plastic, or rubber but must be resistant to the test substance. Reversible neoprene gloves attached to the door have been used in some studies to allow handling of the test animals in the chamber without opening the door (Timbrell et al. 1970). At least one observation port should be included, either at the back of the chamber or in the front of the top pyramid.

In addition to observation ports, access is needed for sampling and monitoring of the test atmosphere. These sampling ports may be specially designed inserts or merely holes cut into the observation port or door; they should be sealed with rubber or PVC stoppers. Hinners et al. (1968) recommend specially designed ports located at the back of the chamber at various heights. These are 1.3-cm diameter stainless steel full couplings with PVC plugs through which steel sampling probes or other monitoring equipment can be inserted into the test atmosphere. Further discussion on sampling equipment is presented in Section 4.3.5.

An important part of the chamber design is the construction and arrangement of animal cages within the chamber. In general the following conditions for cages should be followed whenever possible: (1) for exposure to aerosols, animals should be housed individually in separate cages; (2) larger animals should be placed in the bottom tier of cages; (3) no pans or solid surfaces should be used in cage designs; (4) a layer of open space should separate each cage layer; and (5) cage positions should be rotated to maintain equal exposures for all animals (Fraser et al. 1959). The individual cages should be made of a mesh material, most frequently stainless steel. Some recommended sizes and materials for the cages are: 46 × 46 × 46 cm of stainless steel mesh for dogs and monkeys, 23 × 23 × 38 cm of 1.3-cm mesh hardware cloth for rabbits, and 7.5 × 7.5 × 15 cm of 0.6-cm hardware cloth for small rodents (Fraser et al. 1959). If the test animals are to be continuously exposed, larger cage sizes may be necessary, such as those recommended by the U.S. Department of Health, Education, and Welfare (1974). The small rodent cages can be combined into a single tray of 20 or so components to aid in handling (Fraser et al. 1959). If the chamber door is large enough, the cages can be mounted on a cart, which can then be rolled in and out of the chamber as needed. If this is not possible, cage supports should be added to the chamber walls. These can be metal pins welded into the corners at six levels 25 cm apart or metal tracts mounted on the chamber sides (Hinners et al. 1966). Often metal grates or mesh partitions are used to support the smaller rodent cages. An interesting modification of these standard cage arrangements has been reported by Wells (1979) and Carney (1979). This consists of a "ferris wheel" type arrangement (Figure 4.6) that allows the cages to be rotated automatically during exposure. Such a cage arrangement can help stabilize and equalize exposure, especially for dust exposures, by eliminating shadow effects from the cages. It can also be used to increase the number of animals per chamber (Carney 1979). A variation of this ferris wheel design was discussed by Doe and Tinston (1981); air enters the chamber at the top front, flows in a cyclical pattern concurrent with the rotation of the cage wheel, and is exhausted at the top rear of the chamber. Moss and Brown at Battelle Pacific Northwest Laboratory designed a chamber modification with another alternative cage arrangement (Beethe et al. 1979). This design provides constant concentrations and size distributions of aerosols within the chamber as well as reduced handling time for the animals. The cages are constructed in compartmental units that occupy one-half of the cross section of the chamber. The units are arranged at six different heights and are designed to operate with waste catch pans in place (Figure 4.7). Air enters the top of the chamber, flows down the sides, and is diverted into distributing eddies by the catch pans. However, verifying studies showed that lung deposition in rats varied somewhat for different locations in the chamber. Griffis et al. (1981b) tested the chamber design and found that animals on tiers 2, 4, and 6 (Figure 4.7) had lung burdens 8 to 11% greater than animals on the other tiers. Also, animals on the lower tiers had burdens 5 to 6% greater than animals on the upper tiers. Griffis et al. recommended that animals be rotated among the tiers for chronic tests.

Other design considerations include heating and cooling of the chamber, which is usually accomplished through the air supply (see Section 4.4). Light can be provided by indirect sunlight or preferably by artificial illumination. If artificial illumination is used, light fixtures should be placed outside the chamber to avoid the necessity of specially sealed fixtures (Fraser et al. 1959). Equipment and techniques for maintaining the sanitary conditions in the chamber are discussed in Section 4.3.6.

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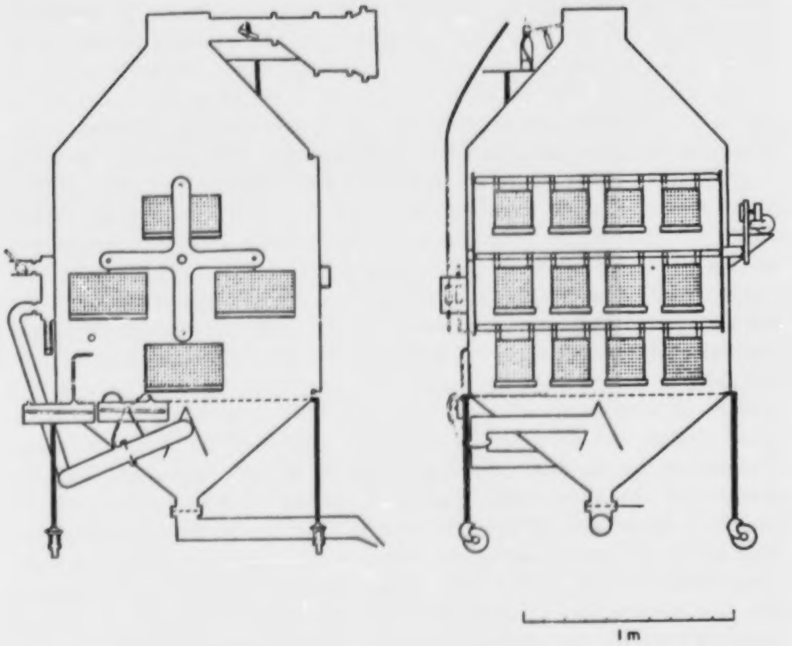

Figure 4.6. Whole-body dynamic exposure chamber of 1.3-m side. The animals are accommodated in 16 mesh cages that are supported on the ferris wheel. (Source: Adapted from Wells 1979. Used with permission of Blackwell Scientific Publishers, Ltd.)

4.3.4 Airflow Systems

The airflow system of an inhalation exposure chamber should include the following components: a source of air, several banks of filters (for both supply and exhaust lines), air conditioning units, a system of ducts, lines, or other containers for distribution, and devices to control and measure the flux and/or amount of air, test substance, and final mixtures (Lodge 1968). There are many ways of incorporating these features into the airflow system. Use of common air supply and exhaust lines is recommended to minimize costs (Fraser et al. 1959). The system should be designed with flexibility of use in mind, since individual components of the system may have to be modified or moved to allow testing of varied substances. A schematic diagram of a "typical" airflow system for dynamic, whole-body exposure is presented in Figure 4.8.

Control of the airflow within the system relies on the proper positioning of values, meters, and pumps to determine its rate, distribution, and

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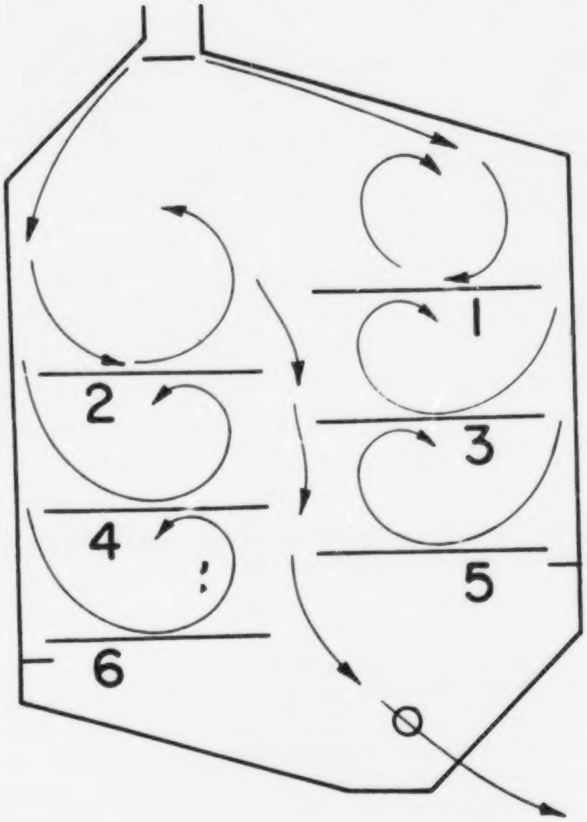

Figure 4.7. Cage arrangement and chamber design of Moss and Brown showing airflow pattern. (Source: Adapted from Beethe et al. 1979. Used with permission of the publisher.)

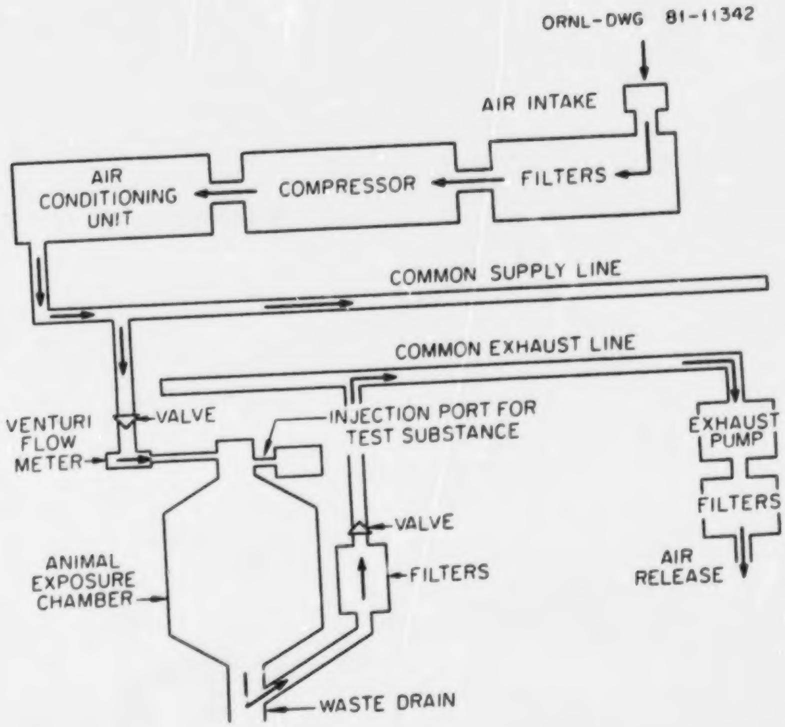
ORNL-DWG 81-11342
AIR INTAKE
AIR CONDITIONING UNIT
COMPRESSOR
FILTERS
COMMON SUPPLY LINE
COMMON EXHAUST LINE
VENTURI FLOW METER
VALVE
INJECTION PORT FOR TEST SUBSTANCE
EXHAUST PUMP
FILTERS
VALVE
ANIMAL EXPOSURE CHAMBER
FILTERS
AIR RELEASE
WASTE DRAIN

Figure 4.8. Schematic diagram of airflow system. (Source: Adapted from Wright 1957 and Wehner et al. 1972.)

operational pressure. An integrated airflow control system was designed by Koizumi and Ikeda (1981) that automatically controlled exposure concentrations, flow rates, and sampling for vapor atmospheres. Their system consisted of four exposure chambers with vapor generators, a tank of methane gas for calibration, a gas liquid chromatograph, a computer with a printer, and a flow controller. Depending on the computer program, a variety of atmosphere types can be generated, including continuous exposure at a given and constant concentration; mixed vapors exposure; exposures with commanded geometric means and standard deviations; wave patterns; and repeated short-term peaks. This variety allows the researcher to duplicate real-life exposure situations, such as variable concentrations of workplace atmospheres, with low variability (coefficients of variation of 3.7 to 6.1%) and with greater reproducibility.

The air pumped into the exposure chamber is generally taken from the room atmosphere (Hinners et al. 1968) or from a central pressurized laboratory supply line (Fraser et al. 1959, Timbrell et al. 1970). These sources provide "pure air," which can be operationally defined as air free of anything known to interfere with the study (Lodge 1968). Of course, to do this, the internal supplies of air must process air from the environment through filtration, compression, and conditioning units (Figure 4.8.).

The rate that the air is delivered through the system is limited at the lower end by the amount of air needed to overcome heat buildup generated by the animals and on the upper end by the atmosphere generation or feed rate (Fraser et al. 1959). For most exposure situations, a rate for small chambers of 2 to 10 ft^3/min (0.6 to 3 m^3/min) or for large chambers of 2 to 20 ft^3/min (0.6 to 6 m^3/min) is recommended (Roe 1968). A venturi-type flow meter, placed in the supply duct and hooked to a magnehelic pressure gauge, is used to monitor the rate (Fraser et al. 1959). Various types of valves have been used to control the rate, including sliding gates mounted on, or geared cones inserted into, orifice plates in the supply and exhaust lines (Fraser et al. 1959); a butterfly damper in the supply line and a globe valve in the exhaust line (Hinners et al. 1966); and flexible pinch valves in the exhaust line (Fraser et al. 1959). The valves should be placed downstream from filter units to prevent clogging (Drew and Laskin 1973). It is necessary to verify the accuracy of the construction and the calibration of valves and meters after actual placement in the system (NAS 1977). Alarms can also be installed in the airflow system to signal when valves, pumps, or other apparatuses have malfunctioned and disrupted the airflow system (Schreck et al. 1981).

The distribution of the air (and test substance) in the exposure chamber must avoid a direct flow from inlet to outlet and areas of stagnant air (Fraser et al. 1959). Criteria for an acceptable chamber exposure were proposed by Carpenter and Beethe (1978) and include: (1) nonrotational uniform flow from inlet to outlet, (2) no aerosol recirculation from below to above animal levels, and (3) uniform aerosol size distribution and concentration distribution. The pyramidal top provides an area for mixing of the atmosphere and works quite well when the air enters tangentially to the short cylinder at the top of the pyramid (Hinners et al. 1968). The test substance is injected similarly (Figure 4.8). However, there is a tendency for the inlet air to flow down the sides of the chamber instead of uniformly. A venturi-type mixing section attached to the top of the pyramid has been suggested as a remedy (Fraser et al. 1959). The air is usually exhausted from the bottom center of the chamber through one arm of a Y joint (Roe 1968). It is just as important to maintain uniform flow for air leaving the chamber as for air entering. A distribution plenum inserted below the exposure zone of the chamber has been recommended to provide this uniform exhaust flow (Fraser et al. 1959, Wehner et al. 1972). Another exhaust alternative producing uniform exhaust flow uses several exhaust ports spread over the bottom of the chamber leading to one central exhaust (Carpenter and Beethe 1978). Modifications of the standard chambers often include airflow alterations. For example, Holmberg and associates modified the Rochester chamber to handle dense diesel aerosols by adding a dispersing cone and screens to produce a more laminar airflow (Holmberg and Moneyhun 1980, Holmberg et al. 1981). Another variation in airflow design involved a switch to horizontal, nearly laminar airflow (Hemenway and MacAskill 1982). This design used a standard rectangular tank with a unique inlet and outlet baffle/plenum to create an evenly distributed flow through the chamber. A prechamber mixing tube allowed the test substance to be blended at the proper concentration prior to injection into the chamber. Fecal pans set below the cages prevented the flow from being totally laminar, but particle size samples indicated no variation in size distribution. The horizontal laminar flow was also used in a chamber design by Ferin (1978). This design used four modules, with one module for filters, the second for injecting the test atmosphere (using perforated tubes), the third containing the animal cages and a perforated plate (diffuser) to convert the airflow from turbulent to laminar, and the fourth containing an exhaust manifold.

Among the various designs mentioned, one study was found that compared the efficiency of their airflow systems. This study used a model to calculate from test data the amount of dead space and turbulence in the design (Hemenway et al. 1982). The model was derived from the mass balance of the system and incorporated a dispersion coefficient as representative of the amount of turbulence. The results showed that a conventional design had 84.5 to 89.5% dead space and a negative dispersion coefficient (often the tracer profile appeared at the outlet point before it showed at the inlet point). The horizontal flow design had dead space areas of 34 to 37% and a dispersion coefficient of 0.14 m^2/s. The multi-tiered chamber (Figure 4.7) showed almost no dead space (0 to 6%) and had a dispersion coefficient of 0.12 m^2/s. These tests were done without the presence of animal cages. Dead space increases time to concentration equilibration at start-up and shutdown, decreasing the utility for pharmacokinetic studies.

The operational pressure of the exposure chamber is controlled by the valves and meters placed in the airflow system. It is generally recommended that the chamber be operated at a negative pressure (relative to the external atmosphere) of 0.25 to 1.3 cm of water (Hinners et al. 1966, Roe 1968, USEPA 1982). This pressure is maintained by exhausting air through a pump in the exhaust line at a faster rate than the rate of incoming air (Fraser et al. 1959). A pressure of 15 to 28 cm of water at an airflow rate of 1000 to 2000 ft^3/min (305 to 610 m^3/min) in the exhaust line will provide the necessary suction for a negatively pressurized chamber (Roe 1968). As a result of the negative chamber pressure, precautions must be built into the valve control system to prevent asphyxiation of the test animals if the air supply in interrupted (Wright 1957).

Conditioning of the air in the exposure chamber is a primary function of the airflow system. This includes maintaining proper temperature and humidity levels and removing any impurities by filtration. Temperature of air flowing through the exposure chamber is affected by temperature of air from the external source, temperature of air inside the exposure building, and heat released by the animals. Bernstein and Drew (1980) have shown that, when intake air is at room temperature, the majority (95%) of excess heat is radiated through the chamber walls. Cooling of the air stream allowed more heat to be removed. With low animal loading of the chamber (eight rats in a 380-L chamber) 40 to 80% of the excess heat is removed by the cooled airstream, but as more animals are used, only 17 to 30% of the animal heat is removed by the airflow. Thus, if high animal loading is necessary and high flow rates cannot be used, then the exposure room temperature should be lowered to provide the necessary cooling to maintain proper chamber temperatures. By maintaining the exposure room and chambers in the same temperature range, heat transfer between these two areas, and radiant heat exchange between the chamber walls and nearby animals are avoided (Fraser et al. 1959). The recommended temperature range for exposure chambers and rooms is based upon preferred temperatures of the most frequently used test species and is 75 to 82°F (24 to 28°C) (Fraser et al. 1959, Hinners et al. 1966). The proper temperature is even more important in inhalation studies because it can affect respiration rate and pattern and thereby the extent of exposure. Fraser et al. (1959) discussed the calculations necessary to select the proper cooling unit and recommended a 1/2-ton refrigeration unit for chambers of 145 ft^3/min (44 m^3/min). If extremely high flow rates (>50 ft^3/min or >15 m^3/min) are used, a 3/4-ton unit is suggested. If the local climate does not require such a large cooling capacity, then copper cooling coils can be wrapped around the incoming air ducts to provide the proper temperature (Hinners et al. 1966). In either case the thermostat should be located in the exhaust line.

Similarly to the detrimental effects on test animals of high temperature, excessive humidity can affect inhalation exposure and, depending on the test substance, test agent concentration. It is necessary to know the heat load and the moisture generation rate of the animals in order to determine the proper controls for humidity. Fraser et al. (1959) discuss the necessary calculations to determine these factors. Frequently, the cooling process will reduce the humidity to the desired levels; however, it may also be necessary to cool the incoming air to below the desired temperature and then heat it to remove excess humidity and provide the correct temperature level. The desired humidity levels are generally accepted as equal to or lower than 55% relative humidity (Fraser et al. 1959). EPA guidelines require a humidity of 40 to 60% (USEPA 1982), but exceptions can occur if the test agent makes this difficult.

Another element of chamber atmospheres that can be a confounding factor is the presence of ammonia. This ammonia comes from the expired air of the test animals (Barrow and Steinhagen 1980) or arises from the action of bacteria on urine and feces of test animals. Elevated ammonia concentrations can react with test chemicals (Barrow 1978) and affect the health of test animals (Weissman et al. 1980). Several studies evaluated the potential impact of ammonia in whole-body chambers and nose-only exposure systems. As expected, ammonia concentrations increased with time. Several mechanisms could be used to acceptably control the levels, including use of bacteriostatic cageboard (impregnated with neomycin); an increase in airflow rates; frequent changing of cageboard; and frequent removal of animal wastes (Barrow 1978, Weissman et al. 1980). A recommended procedure would be to use an airflow rate equivalent to 14 chamber volumes per hour, use bacteriostatic cageboard, change the cageboard daily, and clean the chambers and cages weekly; this should keep levels below 2.7 ppm for mice (Weissman et al. 1980).

To remove potentially interferring impurities and achieve the "pure air" necessary for inhalation studies, it is widely recommended that several banks of filters be employed in both supply and exhaust stages (Fraser et al. 1959, Hinners et al. 1968, Roe 1968). The most common impurities are water vapor and oil droplets (Wright 1957). However, potentially more harmful substances can be found in most air supply sources, and filtration should be designed to handle a wide range of pollutants. Lodge (1968) recommends the use of several filters of increasing efficiency, including charcoal beds to remove organics and polar gases, a furnace for combustion of trace organics, and a final filter to remove particulates from the furnace. Drew and Laskin (1973) recommend filtration through an absolute (HEPA) filter and charcoal beds. Most recommendations require at least two different filters for incoming air, with the first filter to handle large materials (Hinners et al. 1968, NAS 1977). The air with traces of test agent leaving the exposure chamber must also be thoroughly filtered, preferably at both the individual chamber exhaust and the centralized exhaust line (Roe 1968, Drew and Laskin 1973). Timbrell et al. (1970) recommend the use of two primary filters followed by one absolute filter for each chamber exhaust. Drew and Laskin (1973) recommend filtration of chamber exhaust through a roughing filter (to remove animal hair and debris), an absolute filter, and a charcoal filter. They also recommend a similar filter composition for the central exhaust. In special circumstances it may be necessary to use scrubbers, electrostatic precipitators, or cyclones (Drew and Laskin 1973). As an added precaution, Timbrell et al. (1970) recommend high release stacks to help dilute the released exhaust air in the environment.

4.3.5 Sampling and Monitoring of Chamber Conditions

During operation of the exposure chamber, it is necessary to sample the atmosphere inside the chamber to verify that correct exposure conditions are being maintained. Environmental factors that should be monitored include temperature, humidity, pressure, and certain gas concentrations. In addition, the test agent distribution and concentration and the airflow rates must be monitored (USEPA 1982).

The temperature of the chamber can be monitored by mounting a thermometer inside the chamber or by installing, in the chamber wall, thermocouples that are connected to dial thermometers outside the chamber (Fraser et al. 1959, Timbrell et al. 1970). The latter method is preferred when large chambers are used with samples taken from various locations in the air system and exposure chamber (Figure 4.9.). This sampling pattern can also be used to determine humidity and to verify adequate air mixing and distribution within the chamber. Humidity determinations can be obtained by remote probes connected to a hygrometer or by a hair hygrometer placed inside the chamber (Timbrell et al. 1970). Nelson and Taylor (1980) have described an integrated, automatic system that monitors and controls chamber temperature, humidity, and airflow rate. The system uses a commercially built temperature-humidity indicator with a specially designed airflow-temperature-humidity control module. The monitors provide feedback control for the system and, once calibrated, the system provides flow rates with variations of 0.2%, temperatures with variations of 0.1°C, and humidities with variations of 0.5%. This type of automated system requires less operator time and produces excellent control. Correct operational pressures can be monitored by magnehelic gauges connected to the chamber, exhaust line, and supply line (Drew and Laskin 1973). The concentrations of certain gases (e.g., oxygen, carbon dioxide) may need to be monitored occasionally; standard chemical analyses have proved satisfactory for this monitoring (Fraser et al. 1959). For all these monitoring techniques, the accuracy of the procedure and equipment should be periodically verified using reference standards (Fraser et al. 1959).

The main monitoring effort is normally focused on test agent concentration and distribution, using different techniques for gases and aerosols. In general, to determine concentrations accurately, you must sample the actual concentrations in the chamber and at the breathing zone of the animals (Figure 4.9.) and not rely on theoretical concentrations calculated from rate of airflow and test substance feed rate (Fraser et al. 1959). Even if concentrations are measured accurately, there will be variation in the dose calculations as a result of variations in the precision of the analytical method. Also, with most monitoring techniques, there is a delay between sampling and analysis that can limit its applicability (Fraser et al. 1959). In systems that use a blower-powered chamber exhaust, a potential problem is dilution of the test atmosphere by withdrawing air for the sample. If a blower is used to withdraw the sample, the test agent concentration can be affected; to avoid this dilution the discharge from the sampling blower should be routed into the chamber discharge in front of the exhaust blower. This will ensure that the total amount of air withdrawn from the chamber remains constant (MacFarland 1981). Automatic sampling techniques can be used, especially where chemical or photoelectronic methods are employed. These are usually cheaper in the long run, produce a permanent record of exposure, and if rapid responses are possible can be

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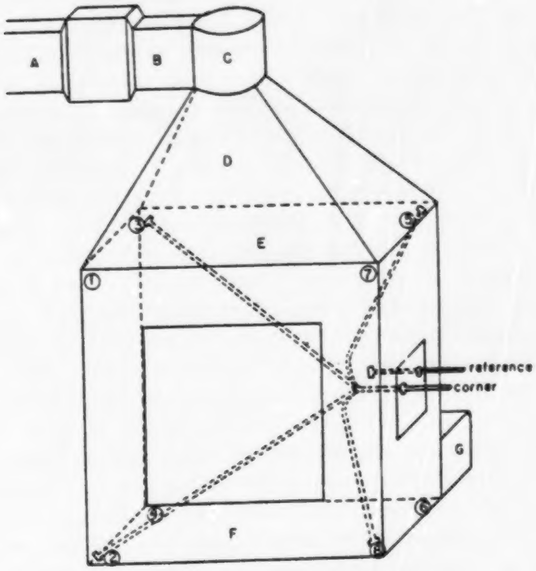

Figure 4.9. Sketch of chamber indicating the eight corner and one reference sampling positions. Letters indicate positions of thermocouples used for measurement of temperature distribution within chamber. (Source: Adapted from Fraser et al. 1959.)

linked to a feedback method for controlling chamber feed rates (Fraser et al. 1959). Currently, the number of automated procedures is increasing (Hinners 1978, Van Stee and Moorman 1978, Kennedy and Trochimowicz 1982), but those in operation should still be verified by calibration or by nonautomated procedures to ensure representative sampling and instrumental accuracy (Lodge 1968, Tillery et al. 1976, WHO 1978).

Sampling techniques are comparatively simple when the test agent is a gas or vapor. One technique is to obtain a definite volume of the test atmosphere at a known temperature and pressure in a container (e.g., evacuated flask). An alternative method is to pass a known volume of test atmosphere through a suitable collective material (e.g., impregnated paper). The samples obtained can then be analyzed by standard analytical techniques (Drew and Laskin 1973). Direct methods for measuring gaseous concentrations can also be used and adapted for automatic analysis (Table 4.6) depending on the test agent. Automatic techniques for sample analysis should be selected based on their specificity, sensitivity, reliability, response time, and cost (Bryan 1970). Nader (1971) reviewed the instrumentation aspects of these continuous monitors.

Table 4.6. Measurement Principles Used in Continuous Monitoring Instruments for Gases and Vapors

Absorption spectrometry	
Visible and ultraviolet	O_3, NO_2, SO_2, CHO^-
Infrared	CO, hydrocarbons
Chemiluminescence	O_3
Electrometric	
Conductometry	SO_2, acid gases
Coulometry	O_3, SO_2, and other electroreducible and oxidizable gases
Ionization (flame)	Organics
Electron capture[a]	Peroxyacylnitrates, halogenated organics

[a] Semiautomatic sequential analysis with gas chromatography.

Source: Adapted from Bryan (1970).

Sampling and monitoring of aerosol and dust exposure studies are more difficult, because both mass concentration and particle size must be determined. Some of the varied sampling methods used for aerosols are listed in Table 4.7 with the particle size range for which they are appropriate shown in Figure 4.10. Sampling methods and equipment for mass concentration and particle size determinations are essentially of three types. One type of sampler roughly separates particles into two groups representative of the upper and lower respiratory tract, another type is designed to separate the test particles into several classes of different sizes or aerodynamic behaviors; and some samplers collect or analyze the particles without any physical separation based on size.

The first aerosol sampling devices were designed to mimic the natural separation of particles that occurs between the upper and lower respiratory tract. Generally, particles of 7 to 10 μm diam are considered to be nonrespirable and represent the particle size found in the upper respiratory tract (Hatch and Gross 1964, Roe 1968). Gravity sedimentation was one technique used to simulate this separation pattern. With laminar flow of air through a horizontal channel, particles will settle out depending on their size and settling velocities (Hatch and Gross 1964). Wright (1954) designed a collector (Hexhlet sampler) using this principle with an elutriator composed of two banks of horizontal aluminum plates separated by a

Table 4.7. Particulate Sampling and Characterization Apparatus

Apparatus	Quantification	Remarks
	Sedimentation	
Dust jars, screens, plates, boxes	Count, weight, chemical analysis	Simple, inexpensive, readily available. Accepted method for pollen. Long settling time for submicron particles. Not isomatric
	Impingement	
Cascade jet impactors	Count, chemical weight, micrurgic	Rapid aerosol fractionation. If to count, not good for high concentrationa. Can calibrate for EAD
Single jet impactors	Count, chemical weight, micrurgic	Efficient to 1 μm, then less efficient. Distinguish solid from liquid aerosols
Fine-fiber impinger	Count, chemical, micrurgic	Simple, compact. Solid-liquid distinctions
Greensburg-Smith impinger	Count, chemical	Soluble gases and particulates. Commercially available, portable
	Filtration	
Glass, asbestos, metal cellulose, and other esters in porous membranes	Weight, chemical, visual, etc.	Wide range of aerosols and applications. Inexpensive, readily available (e.g., Millipore, Gelman)
Paper tape high-volume	Chemical, color, light transmission	Smoke and aerosol density
	Centifugation	
Cyclones	Weight, chemical, count	Wide range of sizes, materials, utilities. Available, inexpensive
	Optical	
Light scattering devices	Particle count concentration and size fractionation by light scattering, tyndallometry	Commerially available. Expensive. Need careful calibration and operation. Automatic, rapid, small sample requirements
Direct microscopy	Count, visual examination, sizing	On impaction, filter, or electrostatically collected samples
	Electrostatic thermal	
Precipitators	Count, micrurgic, visual	Electrocharge, thermal gradient principles. Submicron particles

Source: Adapted from Campbell (1976).

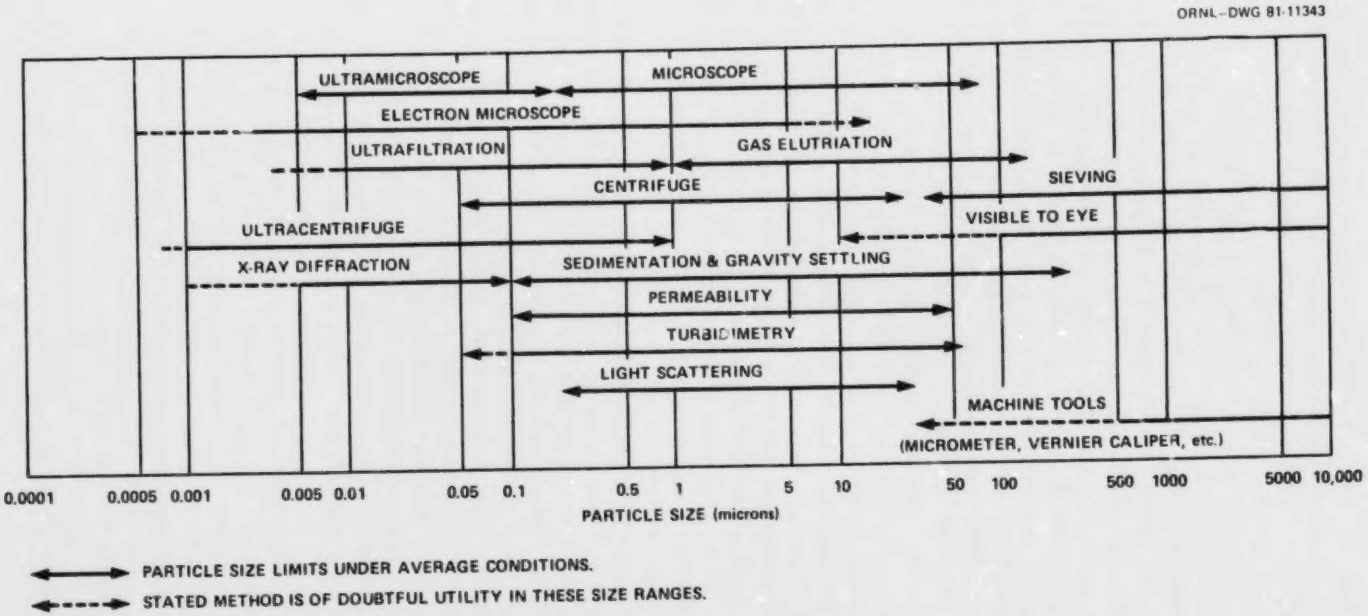

Figure 4.10. Limits of particle size measuring equipment. [Source: Sheehy et al. 1967, as reported in Campbell 1976. Reprinted from *Clinical Toxicology* 9(6):863 (1976) by courtesy of Marcel Dekker Inc.]

vertical partition into 120 inlet ducts for the first separation stage and a soxhlet filter to collect the "respirable" particles in the second stage (Figure 4.11). This unit will collect about 1 g of particles when operated at 100 L/min for 8 h (Hatch and Gross 1964).

Another two-stage collector, the conicycle, uses centrifugal force to both reject the coarse upper respiratory tract particles and collect the fine respirable particles (Figure 4.12). In Figure 4.12, large particles are rejected at A and small particles enter B with deposition on surface C. Very small particles may be partially deposited or exit through orifices O. The sampler operates at a rotational speed of 8000 rpm and a sampling rate of 10 L/min (Wolff and Roach 1961 as cited in Hatch and Gross 1964). Performance curves for the conicycle and hexhlet samplers are shown in Figure 4.13. A two-stage continuous flow cascade impactor that produces a separation window between 4 and 1 μm from a polydisperse aerosol has also been developed (Gat 1980). Other two-stage samplers include the cyclone separator, which uses centrifugal force to separate large particles and a filter to trap small particles, and the preimpinger, which uses changes in air direction and water filled-glass flasks to separate and collect the different particle sizes (Fraser et al. 1959, Hatch and Gross 1964). Generally, there is little difference in collection efficiency between these methods, except for the potential for disaggregation of large particles or aggregations with the cyclone or preimpinger (Hatch and Gross 1964). These differences can be important, because the particles must be collected in the form occurring in the aerosol cloud to be valid particle size determinations.

Aerosol samplers have also been designed to produce multiple stages of particle separation based on size or aerodynamic behavior. The cascade impactor (Figure 4.14) was the first sampler designed with this capability (Hatch and Gross 1964). It consists of a series of impingement slots (usually 5 to 10) arranged in order of decreasing width and distance followed by a high-efficiency filter. It works on the principle that progressively finer particles are deposited at each stage of impingement as a result of increasing jet velocities and decreasing jet dimensions (Hatch and Gross 1964). A commercial model and its particle size separation series are shown in Figures 4.15 and 4.16. The separation series compares rather well to lung separation patterns with, stages 1 and 2 corresponding to nasal chamber deposition, stages 3 to 5 corresponding to upper respiratory airways and pulmonary air spaces deposition, and stages 6 to 8 corresponding to lung deposition. Thus it can be used to identify different sites of deposition for different size particles. The cascade impactor does have two main drawbacks. First, it tends to break up aggregations of particles that would deposit as large particles, and, second, only a limited amount of material can be safely collected on each surface before the risk of dislodgement becomes a factor (Hatch and Gross 1964).

A variation of this sampler is the Electronic Cascade Impactor (ECI). It was designed by Tropp et al. (1980) to provide the following desired

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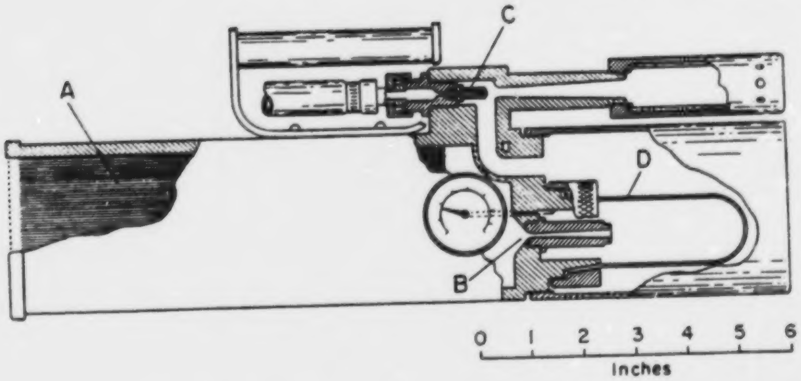

Figure 4.11. Hexhlet two-stage aerosol sampler. *A* = First stage of gravity elutriator with size separation based on the equation $P = 1 - d^2/50$, where *P* = percentage penetrating and *d* = diameter of unit density sphere in microns; *B* = tapered critical orifice; *C* = ejector to maintain a constant airflow rate; and *D* = soxhlet filter. (Source: Adapted from Wright 1954. Used with permission of the publisher.)

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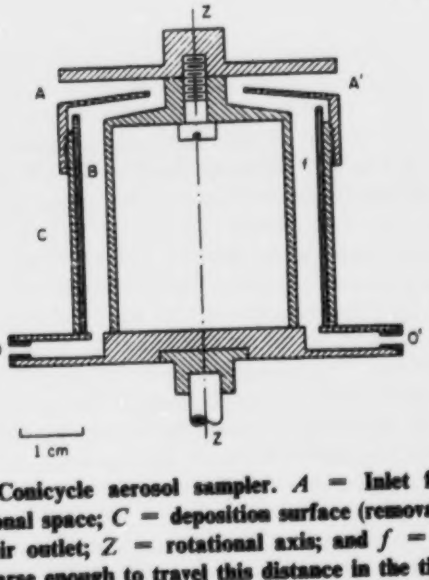

Figure 4.12. Conicycle aerosol sampler. *A* = Inlet for atmosphere; *B* = inner rotational space; *C* = deposition surface (removable) for smaller particles; *O* = air outlet; *Z* = rotational axis; and *f* = radial distance (only particles coarse enough to travel this distance in the time required for the air to pass through the chamber will be deposited). (Source: Adapted from Wolff and Roach 1961.)

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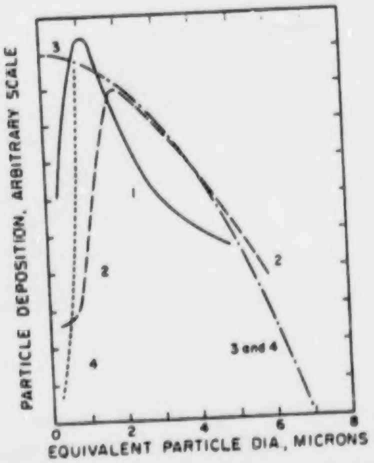

Figure 4.13. Performance curves for the hexhlet and conicycle samplers. 1 = Experimental pulmonary deposition curve of Brown et al.; 2 = Findersen's theoretical curve for human alveolar deposition; 3 = hexhlet calculated performance curve; and 4 = conicycle calculated performance curve. (Source: Adapted from Wolff and Roach 1961.)

characteristics: (1) a response time of one second or less for the entire size spectrum; (2) capability to be calibrated absolutely; (3) capability to measure very dense (without a dilution step) as well as very dilute aerosols; (4) ability to classify (in time sequence, if desired) aerosol particles for subsequent chemical or physical analyses; (5) a large volumetric sampling rate to minimize errors introduced when sampling a spatially heterogeneous aerosol; (6) measurement of the aerodynamic particle diameter rather than such quantities as optical diameter and electrical mobility diameter; (7) low cost in comparison with available instruments; (8) real-time output of data that is easily interfaced to recording or data acquisition equipment. Generally, the ECI operates as does a standard cascade impactor, except that the aerosol particles are given a unipolar electrical charge as they enter, each collection stage is isolated electrically from the other stages, and each stage is connected to an electrometer detector. As particles strike the collection surfaces, they give rise to a current that is used to measure and record the particles. Because each collection stage captures only a certain size range of particles, both the number and size distribution can be determined. Other automatic analysis techniques have also been combined with cascade impactors including piezoelectric sensors and sensors that detect decreases in beta radiation resulting from particle deposition (Smith et al. 1978).

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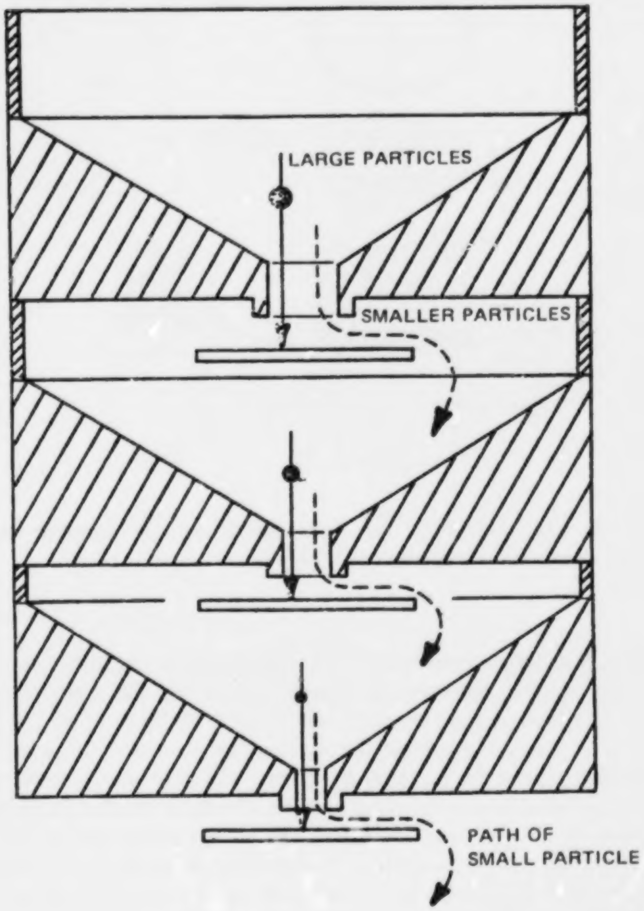
LARGE PARTICLES
SMALLER PARTICLES
PATH OF
SMALL PARTICLE

Figure 4.14. Schematic diagram showing operation of cascade impactor. (Source: Adapted from Smith et al. 1978).

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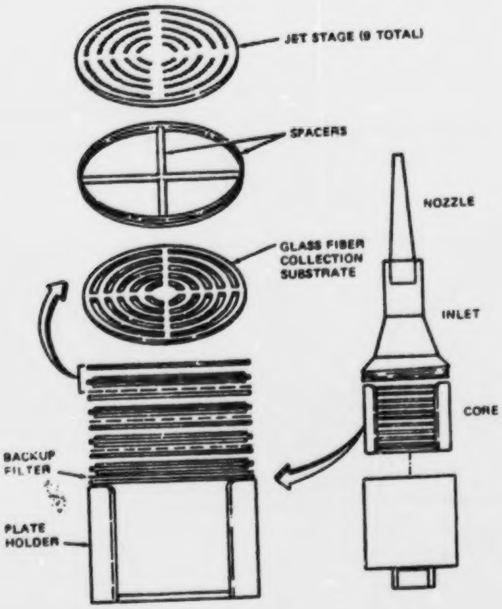

Figure 4.15. Schematic of Anderson Mark III cascade impactor. (Source: Adapted from Smith et al. 1978.)

The collection performance of two cascade impactors and a new instrument, the inertial spectrometer, are compared by De Zaiacomo et al. (1983). The inertial spectrometer was designed by Prodi et al. (1979) and works by injecting the aerosol into a clean air stream above a 90° bend. The bend separates the particles by inertia, and they leave the original air stream at distances as a function of their aerodynamic size (Figure 4.17). The particles then impact on a filter that is the outer edge of the bend. This design allows the particles to be separated while they are airborne, in a continuous distribution, and avoids the problems of bouncing and reentrainment (De Zaiacomo et al. 1983). The two cascade impactors were a multislot design and a multiorifice design with both having eight stages and a backup filter. The comparison found that for smaller size particles the three designs performed equally well. For larger aerosols, the multislot impactor overestimates the median size and geometric standard deviation in comparison with the other two designs.

Another multistage sampler design is the conifuge developed by Sawyer and Walton (1950). It uses gravitational force (similar to the elutriator) with an increase in force supplied by centrifugation. Air is introduced at

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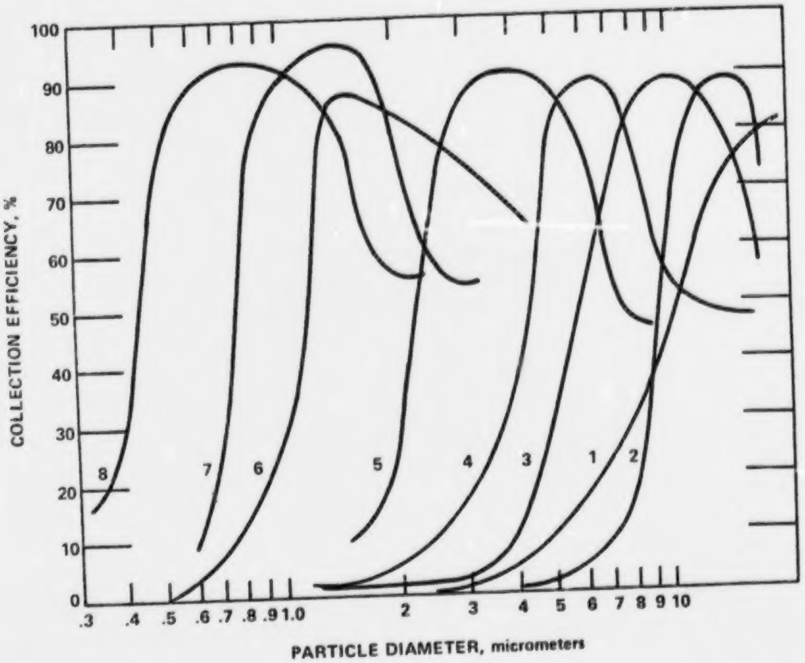

Figure 4.16. Calibration of an Anderson Mark III impactor. Collection efficiency is plotted vs particle size for stages 1 through 8. (Source: Adapted from Smith et al. 1978.)

the apex of a double-walled, hollow, rotating cone, and the particles are deposited on microscopic slides on the outer wall in decreasing order of size from top to bottom (Roe 1968). This design produces excellent size separation without breaking up large or aggregated particles but is limited by a slow sampling rate (Hatch and Gross 1964).

Samplers have also been designed to collect particles without any differential separation. Simple filters and membrane filters are occasionally operated without separators. Precipitator devices can be fitted with some type of separator but are usually operated without them. Optical detection devices are designed to operate without the need for a separator.

Precipitators have been designed that collect particles by thermal or electrostatic methods. The thermal precipitator consists of a narrow channel with a heated wire or surface opposed by a cool collection plate or slide. The particles are struck by molecules with a higher velocity from the heated side and are forced toward the cool deposition surface (Tillery et

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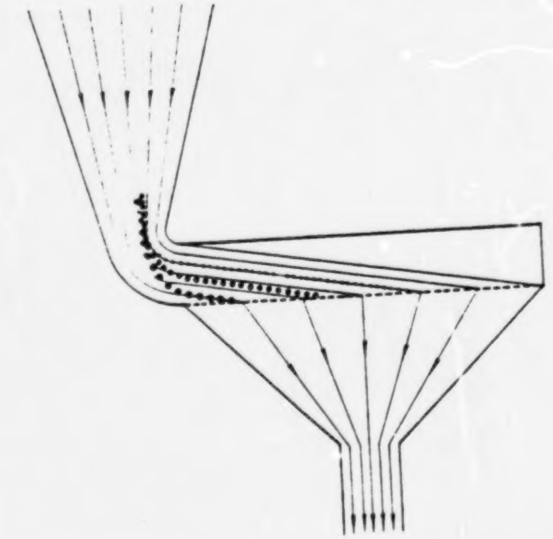

Figure 4.17. Working principle of the inertial spectrometer. (Source: Adapted from DeZaiacomo et al. 1983. Used with permission of the publisher.)

al. 1976). Electrostatic precipitators are usually operated on a point to plane discharge design, with a corona discharge from a needle point charging particles for deposition on a conductive carbon-coated surface (Tillery et al. 1976). An electrostatic precipitator that uses a radioactive tritium source to provide ions for charging particles is shown in Figure 4.18. Precipitators generally operate at low flow rates of 5 to 10 cm^3/min and may occasionally show some size separation.

Optical devices may be used to analyze particle size, mass, and numbers. In general, they operate by using photomultiplier tubes to detect absorbtion or refraction of projected light by particles (Drew and Laskin 1973). The photomultiplier produces a pulse proportional to the size of the particle. One optical monitor uses a laser Doppler velocimeter (LDV) to measure particulate velocity in an acoustic field (Mazumder and Kirsch 1977). A particle entering the sensing field of the LDV is excited by an acoustic wave, and the particle oscillations are monitored. By measuring the phase lag between the oscillation signal and the acoustic wave signal (measured by a microphone) the aerodynamic diameter and number of particles can be determined. A prototype instrument using this technique, the single particle aerodynamic relaxation time (SPART) analyzer, has

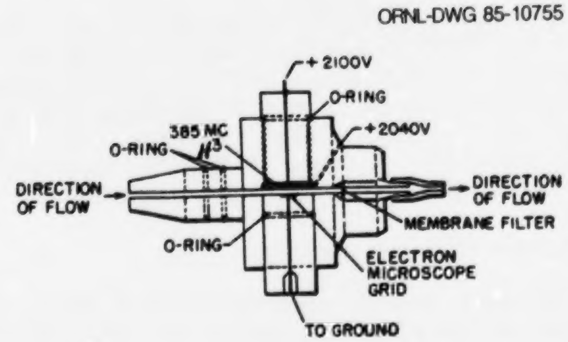

Figure 4.18. Electrostatic precipitator using radioactive tritium (H_3) as an ion source. (Source: Adapted from Tillery et al. 1976. Used with permission of the publisher.)

been developed (Mazumder et al. 1979). It can analyze particulate samples in the size range of 0.2 to 10 μm at a rate of 200 particles per second. Another laser (He:Ne) device for continuously monitoring the mass concentration of monodisperse aerosols was reported by Carlon et al. (1980). Optical devices are most useful when nondestructive or noninvasive techniques for monitoring particle size and number are necessary. However, caution must be used with these devices, because light scattering is not simply a function of size (Tillery et al. 1976); many variables can affect the results (Drew and Laskin 1973). The use of optical techniques to monitor particulates in industrial process streams has been reviewed by Smith et al. (1978). Potential problems with other nonoptical aerosol samplers have also been reviewed. Heindryckx and Dams (1981) investigated some errors associated with cascade impactors used in field situations, and some of their information also applies to inhalation study sampling. Lund et al. (1979) reviewed practical problems arising from the relationship between dust generators and collectors. Formignani et al. (1982) discussed calibration apparatuses for filter media of aerosol samplers.

Except for the optical techniques just discussed, the above sampling techniques must be followed by analysis of the sample. If the sample is obtained on a filter media, various routine gravimetric or analytic techniques (e.g., chemical, UV absorption, chromatography) can be used to determine mass concentration. The filter media to be used is often determined by the analytical technique employed. Microscopic techniques (both light and electron) can be used to determine particle number and size and are most frequently used with samplers that deposit the particles on a removable surface (e.g., an electron microscopic grid in an electrostatic precipitator). Computerized scanning devices can be used to automate analysis of microscopic samples and simplify counting and sizing of particles (Smith et al. 1978). In general, the various sampling and monitoring methods produce comparable results. Halbert et al. (1981) compared particle size distribution analysis using three techniques. They found little difference in analysis results between an electrostatic precipitation–microscopic technique, a cascade impactor–gravimetric technique, and a laser–acoustic relaxation-time technique.

4.3.6 Maintenance of Sanitary Conditions

It is important to maintain sanitary conditions in the exposure room and chambers to avoid potential interference from disease or illness. This maintenance includes both design features of the chamber and cleaning techniques.

To aid collection of test animal wastes, the chamber should have a pitched floor, smooth interior walls, and a centrally located drain (Drew and Laskin 1973). If the chamber is used for continuous exposures, a spray ring is usually installed to wash down wastes. The preferred location is at the top of the bottom pyramid below the metal floor grate (Hinners et al. 1966). The ring is a 1/2-in.-diam stainless steel pipe with four to eight wide-angle nozzles and is controlled by an external hand valve (Hinners et al. 1968). At the bottom of the chamber a 3-in.-diam gate operated by a quick release is used to drain the wash water. This is usually connected to a sewer line, unless the test substance is dangerous enough to require collection in containers (Drew and Laskin 1973). If the chamber is used for intermittent or acute exposures that allow removal of the animals, a spray ring can be installed at the lower edge of the top pyramid to wash down the sides or an external hose can be used (Hinners et al. 1966, Drew and Laskin 1973). To avoid problems with the airflow systems (especially clogging with animal hair), the lines should be free from rough surfaces, restrictions, or sharp bends (Hinners et al. 1968).

During cleaning, it is important to prevent cross contamination between chambers and to protect the health of the technicians. Thus only one chamber at a time should be opened and cleaned (Timbrell et al. 1970). While cleaning, the operators should wear protective clothing and respirators (Timbrell et al. 1970). If a hose is used to wash down the chambers, the water pressure should be low to avoid creating respirable mists of the water-chemical mixture (Smith and Spurling 1974). For chambers with spray rings, the water temperature should be no more than 102°F (39°C), to prevent steaming within the chamber (Hinners et al. 1968). Chambers with stainless steel components must be cleaned frequently to maintain the useful life of the chamber. Cleaning solutions should not remain in contact very long. A cleaning solution of acid detergent with wetting, penetrating, and low-foaming agents has been used successfully for cleaning deposits on stainless steel (Hinners et al. 1968). After all chambers have been cleaned, the exposure room should also be washed down or vacuumed (not swept) to ensure that no contaminants or waste materials remain (Timbrell et al. 1970).

One aspect of maintaining sanitary conditions is the protection of workers associated with the testing procedures. In addition to the steps mentioned above, efforts are needed to ensure that workers are not accidentally exposed to remnant or leaking test atmosphere. Such exposure could occur through loading of the vapor generation system during start-up, leakage during the test procedure, and release during shut-down procedures (Klein and Geary 1982). Automatic sampling of the chamber and test room will assist in determining when hazardous or safe conditions exist. A special problem occurs when particulate substances are being tested, especially particulate carcinogens (Drew 1978). In these cases, workers can be exposed to particulates on the cages, animals, and walls of the test chamber. In addition to dermal contact, these particulates may reenter the atmosphere and be breathed by the workers (Drew 1978). Use of a service module or glove and pass boxes will reduce this potential for exposure. Also, use of and monitoring for an inert indicator substance (such as sulfurhexafluoride) in the aerosol airstream can indicate when small leaks occur. This approach is especially useful when rapid monitoring techniques are not available for the test substance (Moorman 1978). These procedures along with a disease control program will avoid interference with the exposure studies.

4.3.7 Specialized Equipment

In addition to the standard equipment described in earlier sections, specialized equipment is often used in inhalation studies. This includes whole chambers designed for unique exposure conditions as well as equipment designed to test nonroutine conditions of inhalation.

Specialized exposure chambers have been developed by research laboratories of the armed services. These include chambers that operate at pressures higher or lower than normal atmospheric pressure, and systems for studying biological aerosols at low temperatures (Drew and Laskin 1973). Outer space and underwater exploration programs have prompted many of these special chamber designs.

Another special inhalation exposure chamber design for nose- or head-only exposure was discussed in Section 4.3.2.4. A part of this system, the body plethysmograph, is also used for measuring functional alterations in respiration (see Section 4.2.5.4). The body plethysmograph consists of an airtight metal or plastic (Lucite® or Plexiglass®) cylinder designed to contain the animal with its nose and head inserted in the test atmosphere and to allow measurement of pressure changes resulting from breathing movements. For rodents, the cylinder varies in size from 9 to 23 cm in length and from 3 to 12 cm in width (Swann et al.1965, Alarie 1966). The front is usually a combination of straight or curved rigid plastic plates and flexible sheets of sealing material (neoprene rubber, latex, etc.) with a centrally located hole for the head or nose (Thomas and Lie 1963, Murphy and Ulrich 1964). Occasionally, a sealed face mask is used to deliver the test agent and provide the necessary front seal. The cylinder is connected to three tubing lines, one a transducer pressure line to monitor pressure changes, another to conduct the pressure wave from the pump, and a third to bleed off excessive pressure (Murphy and Ulrich 1964, Swann et al. 1965). Dorato et al. (1983) discuss a modification of the body plethysmograph that uses an intraesophageal catheter and an analog computer to produce real-time calculations of pulmonary parameters in rats. This procedure did not require surgery or prolonged anesthesia and was capable of providing multiple evaluations over extended time periods (40 weeks). A body plethysmograph for use with dogs has been described by Boecker et al. (1964) and consists of a large rectangular Plexiglass chamber with an internal sling for restraining the animal and a latex face mask for delivery of the test agent. Although the test animals are usually kept in the plethysmograph for only a few hours at a time, it is best to acclimate them prior to the study by placing them in the container for increasing periods (Smith and Spurling 1974). This will avoid unwanted stress.

Specialized equipment has also been designed to be inserted into the chamber for use. One such type of equipment allows evaluation of inhalation effects on animals that are exercising. For rodents, this consists of a revolving cylindrical wire cage (46 cm diam and 61 cm long) that rotates at a speed of 6.5 rpm and can hold 10 to 15 animals (Fraser et al. 1959). Another version designed by Illing et al. (1975), consists of a wheel 15 cm in diameter and 3.8 cm wide that is open on one side and closed by the converging wires on the other. The cages are mounted on a steel rod run through the center and separated by Teflon® sheets. A small motor rotates the cages via the rod at speeds of 1 to 6 rpm. Mice or hamsters can be tested individually in this design. To test dogs, a rubber treadmill with an effective area of 56 cm by 1.4 m was designed with operational speeds of 0.9 to 7.5 mph (Fraser et al. 1959). Such equipment is not part of a routine study, but would be useful if the expected exposure of humans would involve activity.

4.4. GENERATION OF TEST ATMOSPHERES

4.4.1 Introduction

Testing of chemicals by the inhalation exposure route requires the generation of many types of test atmospheres. The production of an atmosphere of a precisely known composition is one of the most difficult tasks the toxicologist faces because the ratio of agent to air can be one to several million (Lodge 1968). The wide variety of potential atmospheres precludes a universal apparatus suitable for generating atmospheres for all inhalation tests (Clark 1977). Each type of atmosphere must be treated differently, and, even within each type, chemicals with different properties can require unique generation techniques or equipment. The desired objective is to produce the required atmosphere with the most efficient technique available (Carney 1979).

The types of atmospheres generated can be grouped into two broad categories, gases or vapors, and aerosols. Gases and vapors were classified according to their mode of physiological action rather than their structure by Casarett (1975). Asphyxiants are one class of gases and vapors and include both simple and chemical forms. An asphyxiant is any substance that has the capacity to deprive tissues of oxygen by any means other than impairment of respiration mechanics (Casarett 1975). Simple asphyxiants are usually physiologically inert gases (e.g., nitrogen) that prevent oxygen from reaching the respiratory surfaces, while chemical asphyxiants (e.g., carbon monoxide) prevent the body from utilizing the oxygen (Casarett 1975). Irritants are the other major type of gases or vapors and produce inflammation in tissues with which they are in contact (Casarett 1975).

Aerosols include any system of liquid droplets or solid particles dispersed in air with small particle sizes (usually $<10\ \mu m$) and consequently low settling velocities to possess considerable stability as an aerial suspension (Hatch and Gross 1964). Even more than gases or vapors, aerosols exhibit a high interdependence between physical characteristics and toxicity (Casarett 1975). There are many types of aerosols; some of the more common are discussed below.

1. Dusts are fine particles (diameter range of angstrom to $100\ \mu m$) of solid material formed by mechanical disintegration of matter and dispersion into air (Hatch and Gross 1964). They are chemically and constitutionally the same as their parent material (Casarett 1975).

2. Fumes are clouds of particles (diameter $<0.1\ \mu m$) formed by combustion, sublimation, or condensation and are usually accompanied by a chemical change (Casarett 1975). Often this term is restricted to descriptions of droplets from metal oxides of zinc, magnesium, iron, etc. (Hatch and Gross 1964).

3. Smoke refers to a mixture of fine liquid droplets and solid particles ($<0.5\ \mu m$) with liquid and vapor phases produced by combustion (usually of organic material) (Hatch and Gross 1964, Casarett 1975).

4. Mists and fogs also occur in liquid and vapor phases with liquid droplets of any size range formed by condensation of vapors on suitable aerial nuclei or by uptake of water by hydroscopic particles (Hatch and Gross 1964, Casarett 1975).

Each of these aerosol types and the gases and vapors require a unique generation methodology; the following sections will discuss the equipment used to produce such atmospheres.

4.4.2 Generation of Gas and Vapor Atmospheres

The generation of test atmospheres for gases and vapors is relatively simple, involving fewer potential complications than the generation of aerosol atmospheres. Gases and vapors can be tested in a static or dynamic mode. The only requirement is that the method of introducing the contaminant must be compatible with the mode of operation (batch introduction for static systems and continuous feed for dynamic systems) (Lodge 1968).

A commonly used batch method for generating an atmosphere is to mix and store the contaminant in a high-pressure cylinder (Cotabish et al. 1961, Tillery et al. 1976). The gas or volatile liquid is introduced into the empty cylinder by syringe injections or manometer measurements and diluted with air to the desired concentration before release into the exposure chamber. This technique can be used with stable contaminants and, if the release rate is slow, can also be used with a dynamic system. Another simple technique uses high-pressure cylinders containing only the contaminant. The contaminant is metered into a flowing stream of pure air by valves and a calibrated rotometer, which delivers the proper concentration to the exposure chamber (Amdur 1957, Saltzmann 1961). This technique is also limited to stable gases or highly volatile liquids during dynamic exposures. An expansion chamber or reservoir is often used in conjunction with such a system (Carson et al. 1962).

When the gas or vapor is not highly volatile or chemically stable enough to store in high-pressure cylinders, other methods exist to introduce contaminants into a flowing air stream. One of the earliest used techniques for liquids with high boiling points is to inject the liquid through a motorized syringe onto a heated block or into a heated tube which vaporizes it (Carpenter et al. 1949, Tillery et al. 1976). The vaporized contaminant is then introduced into the air stream by a pump or aspiration needle.

A problem with using such direct heating systems to vaporize liquids is the potential for charring at the heat source or thermal decomposition of the sample (NAS 1977). To avoid this problem, two techniques have been recently reported. Potts and Steiner (1980) redesigned a counter-current system to use a perforated-plate distillation column (Figure 4.19). The liquid contaminant is introduced at the top of the column and flows downward. Air is introduced at the bottom of the column and flows upward, becoming saturated with the vapor as it passes over the distillation plates. This system provides a vapor source of constant concentration, without thermal decomposition or condensation resulting from cooling of a heated, saturated vapor. However, the concentration must be measured in the exposure chamber because no direct measurement of the amount of liquid vaporized is possible. Also, since the vapor pressure of low volatile liquids is not accurately known at room temperatures, the experimenter must use trial and error to determine the correct parameters necessary to achieve the desired concentrations. A second new technique to avoid thermal decomposition is designed to handle liquids that are neither highly or

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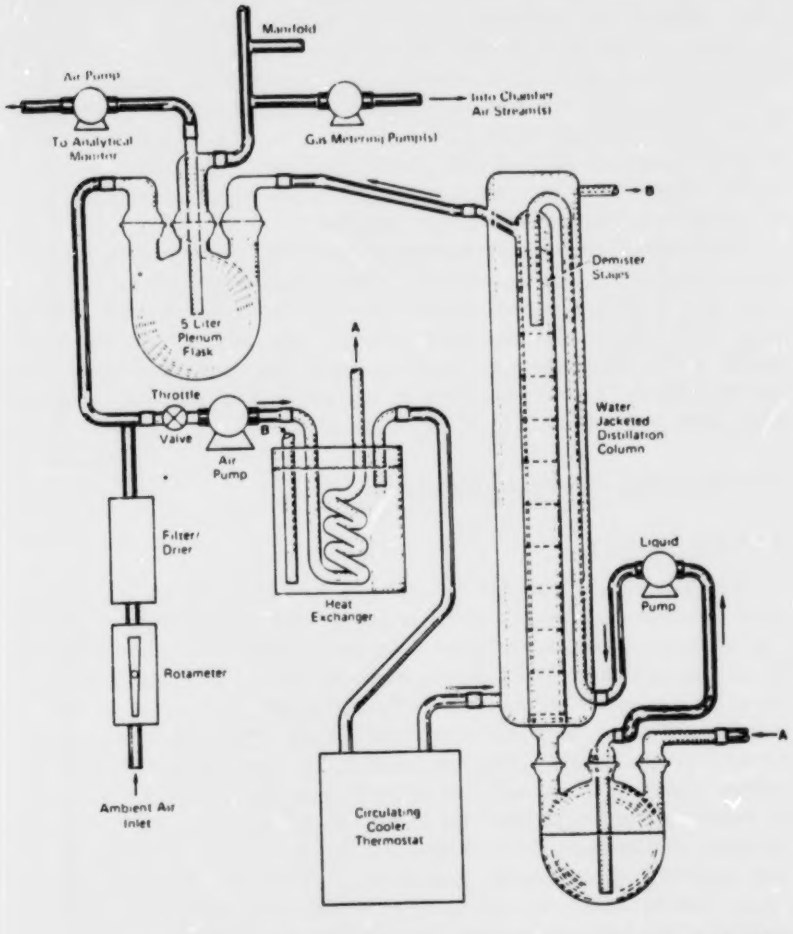

Manifold
Air Pump
Into Chamber Air Stream(s)
To Analytical Monitor
Gas Metering Pump(s)
B
Demister Stages
5 Liter Plenum Flask
A
Throttle
Valve
Air Pump
B
Water Jacketed Distillation Column
Filter/Drier
Heat Exchanger
Liquid
Pump
Rotameter
A
Ambient Air Inlet
Circulating Cooler Thermostat

Figure 4.19. Improved counter-current vaporizing apparatus, with thermostating jacket. (Source: Adapted from Potts and Steiner 1980. Used with permission of the publisher.)

minimally volatile (Miller et al. 1980). A J-tube design vaporizer (Figure 4.20) is filled with 1/4-in. glass beads to increase the vaporization surface. The liquid is introduced at the bottom of the J through a capillary tube. Heated air flows up through the system, vaporizing the liquid as it passes over the glass beads. By preheating the air, chances for charring and thermal decomposition are greatly reduced. This design also provides a greater safety margin when potentially explosive liquids are vaporized.

Another technique for vaporizing liquids is based on two saturation chambers and is similar in principle to the counter-current column of Potts and Steiner (1980). An inert carrier gas is passed through two gas washing bottles containing test liquid, with the first bottle at a higher temperature than the second (Saltzmann 1961, Drew and Laskin 1973). The use of two bottles ensures saturation, but a filtering step following the chambers may be necessary to trap unwanted liquid droplets.

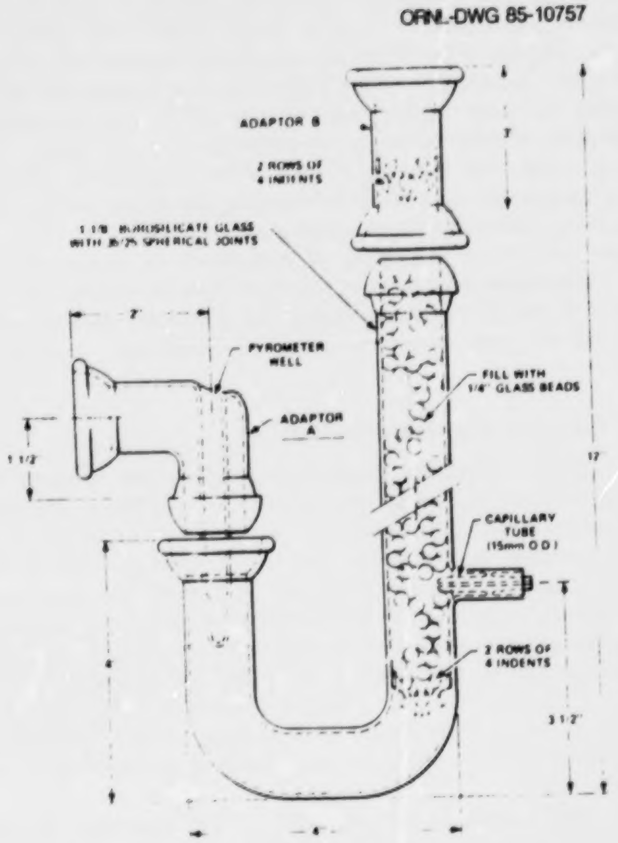
Some unique methods for dispersing vapors and gases are based on diffusion through permeable materials. In one system vapor diffuses at a constant rate through a porous asbestos plug (Lodge 1968). Certain plastics (e.g., Teflon®) have also been used (O'Keeffe and Ortman 1966, Drew and Laskin 1973). These systems require constant flow and temperature. The rate of diffusion can be controlled by varying wall or plug thickness and area (Drew and Laskin 1973, Tillery et al. 1976).

4.4.3 Generation of Aerosol Atmospheres

4.4.3.1 Introduction

The generation of aerosol atmospheres for inhalation testing involves more variables than the generation of gas or vapor atmospheres. The division of solids or liquids into small particles or droplets and their dispersal in air produces two major changes, increased surface area and total space occupied, that affect the toxicity (Drew and Laskin 1973). These changes intensify the basic chemical and physical activity of the test agent, including its rate of oxidation, solubility, evaporation, and deposition. Also, the particle size that the parent material is divided into determines the depth of penetration and deposition site in the respiratory tract. Therefore, it is necessary to determine the particle-size capability as well as the concentration capability of the aerosol generation equipment in order to select the proper unit for each test. The equipment must produce and maintain the desired mass concentration and the desired mean effective particle size with an acceptable dispersion around the mean (Lodge 1968). Size frequency information is generally not useful for dosage evaluations, so most discussions refer to mass frequency because the mass quanity (mass median diameter) is generally a cube function of the easily measured particle diameter (Drew and Laskin 1973).

Aerosols can be produced in two forms, monodisperse and polydisperse. Polydisperse aerosols include a wide range of particle diameters with the mass median diameter having a large geometric standard deviation and are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ADAPTOR B
2 ROWS OF
4 INDENTS
1 1/8 BOROSILICATE GLASS
WITH 35/25 SPHERICAL JOINTS
2"
PYROMETER
WELL
ADAPTOR
A
1 1/2"
3"
17"
FILL WITH
1/4" GLASS BEADS
CAPILLARY
TUBE
(15mm O.D.)
2 ROWS OF
4 INDENTS
3 1/2"
4"
4"

Figure 4.20. J-tube vaporization assembly. (Source: Adapted from Miller et al. 1980. Used with permission of the publisher.)

considered the normal form for aerosols occurring in actual exposure conditions (Drew and Laskin 1973, Casarett 1975). Monodisperse aerosols are composed of particles with a narrow range of sizes resulting in a mass median diameter having such a small geometric standard deviation that no significant error is introduced into the experiment (Wilson and LaMer 1948, Mercer 1973). Monodispersity of an aerosol thus varies depending on the experimental design or requirements and is an arbitary determination of the experimenter (Mercer 1973). Fuchs and Sutugin (1966) defined it as those aerosols having a geometric standard deviation of 1.22 or less when the particles have a log normal distribution; this definition is reasonable for most applications (Mercer 1973). The advantages of a monodisperse aerosol relate to simplification for the study of factors affecting toxicity and handling of data (Wilson and LaMer 1948). Monodisperse aerosols are also useful for studying deposition and clearance patterns and for calibrating the monitoring equipment (Mercer 1973).

Many techniques and types of equipment for generating aerosols, whether monodisperse or polydisperse, have been developed. The following sections will discuss the major designs for generating aerosols using solid feed materials and liquid, solution, or liquid-solid suspension feed materials.

4.4.3.2 Generation of aerosols from solids

The earliest generating designs for solid or dust aerosols involved dispersion of loose noncompacted particles. Air was blown through a containing device in which the loose dust particles were being kept in suspension by vibrations, rotation of the container, or stirring. These devices produced polydisperse aerosols unless the loose dusts had been previously sorted and sifted to make a monodisperse aerosol. Unfortunately, these techniques did not produce aerosol clouds with very consistent characteristics, due in part to the inability to predict the quantitative effects of changes in their methodology (Wright 1950, Roe 1968). Details of the early (1885-1940) designs can be found in Wright (1950). Later dust aerosol generators of this type have been designed to avoid many of these problems.

Drew and Laskin (1971) designed a dust generator to use with material that is not readily dispersed from the preferred prepacked plug (e.g., polyester-fiberglass dusts). For the fluidizing chamber, they used an inverted 1-gallon glass jar with a 3-in., four-blade fan mounted in the screw cap. Two 3/4-in. access holes were provided in the top of the chamber for an air inlet and an aspirator baffle chamber connection. The dust (either polydisperse or monodisperse) is kept in suspension by the fan and removed via an aspirator. Upon leaving the aspirator, the particles impact on the walls of the Lucite® baffle chamber (mounted directly above the glass fluidizing chamber), which removes the larger particles. The remaining particles are piped into the exposure chamber. The chamber concentrations depend on the initial dust charge, the overall airflow through the chamber, and the aspirator airflow. In addition to providing a generation method for noncompactible dusts, this device also avoids agglomeration resulting from electric charges normally associated with dusts.

Another alternative design for generating aerosols from loose dusts is the fluidized bed generator (FBG). The fluidized bed uses air blown through a porous bottom of a container to put dry particles in suspension. The FBG can be combined with a vertical aspirator, an inverted funnel in the bed (Tillery et al. 1976), or a horizontal eductor to remove the dust particles (Guichard 1976). To produce fine particles, the bed can be constructed from 100 to 200-μm-diam glass beads (Guichard 1976) or 100-μm diam nickel spheres (Willeke et al. 1974). The concentration of the aerosol and the particle size distribution are dependent on the operating conditions of the bed (Carpenter and Yerkes 1980). Bed loading and superficial air velocity influence the mass median diameter and the concentration (bed volume also affects concentration) in such a manner that high bed loadings and volumes and low superficial velocities produce maximum concentrations while moderate bed loadings and high velocities produce the smallest particle sizes. Thus, a trade-off must be made between size and concentration in selecting operating conditions (Carpenter and Yerkes 1980). The FBG has been used to generate fibrous dusts (Spurney et al. 1976, Marple et al. 1978) and shows good potential in this area.

The two systems just described are typical of the recent loose dust dispensing devices. The majority of these use an aspirator to remove the loose particles from various types of fluidizing chambers. In general, this type of aerosol generator is most useful for processing dusts that cannot be successfully compacted and redistributed.

The most successful and widely used technique for generating dust aerosols utilizes compressed plugs of powder that are ground or abraded to release the particles. The Wright dust feed mechanism (Figure 4.21) was one of the first of these designs and is still widely used today. Basically, the design consists of a gear-train assembly powered by a synchronous motor which drives a brass cup packed with dust down a spindle shaft in a rotating motion and against a scraper blade that continuously abrades off a uniform amount of dust (Wright 1950, Fraser et al. 1959). The brass cup is slightly larger than the scraper blade, which is a piston with a spring top having a radial cut edge turned up to form a scraper. Air is blown into the lower part of the brass cup; passes through an air channel under the scraper blade, where it picks up the dust; goes down a hollow shaft that supports the blade; and after leaving the shaft, strikes an impactor plate that breaks up any aggregated particles (Wright 1950, Tillery et al. 1976). If the dust plug is compacted with a consistent density and if 90% of the particles have a diameter of 10 μm or less, there should be no problem in redistributing the dust at the proper size, provided the relative humidity of the airstream is controlled (Wright 1950, Roe 1968, Drew and Laskin 1973). Any fluctuations in dust concentration (e.g., due to buildup

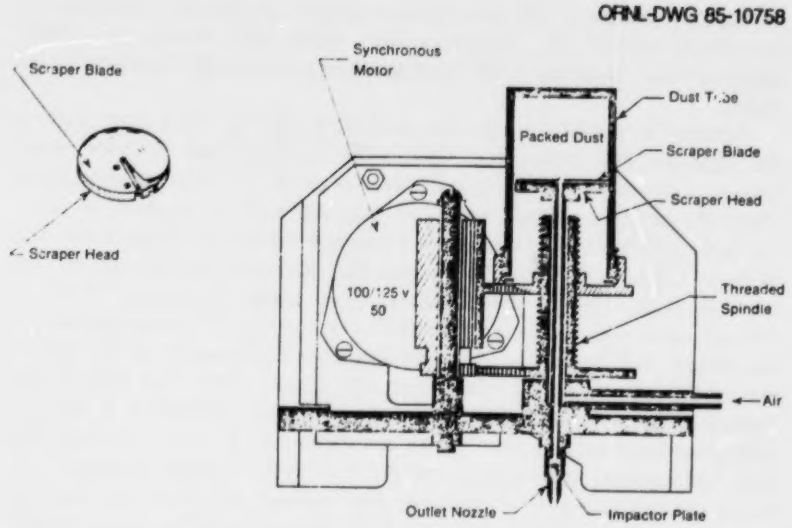
ORNL-DWG 85-10758
Scraper Blade
Scraper Head
Synchronous Motor
Dust Tube
Packed Dust
Scraper Blade
Scraper Head
100/125 v
50
Threaded Spindle
Air
Outlet Nozzle
Impactor Plate

Figure 4.21. The Wright dust feed mechanism. (Source: Adapted from Leach et al. 1959.)

on the scraper blade) can be smoothed out by adding a large reservoir after the impactor.

Another dust generator using a horizontal feed of a compacted powder plug has been designed to handle fibrous dusts (Timbrell et al. 1968). The horizontal feed mechanism consists of a piston-cylinder arrangement with a threaded rod controlling the extent that the powder plug is introduced into the scraper (Figure 4.22). A specially designed rotor driven by an electric motor shaves off a layer of dust inside the dispersing chamber. The particles are carried away by air entering through a side or bottom port and exiting through a port in the top. As with the Wright mechanism, proper packing of the plug and control of relative humidity are needed to produce the desired concentrations. The plug should be compacted all at once to prevent the formation of layers. The Timbrell generator was specifically designed to process asbestos and is capable of separating individual fibers or fiber bundles for distribution. Compacted powder plugs have also been abraded to produce dust particles by using air jets (Dimmick 1959, Hounam 1971).

4.4.3.3 Generation of aerosols from liquids, solutions, and liquid suspensions

More types of aerosol generators exist to create particles from liquids, solutions, and suspensions of solids in liquids than from dust or solids.

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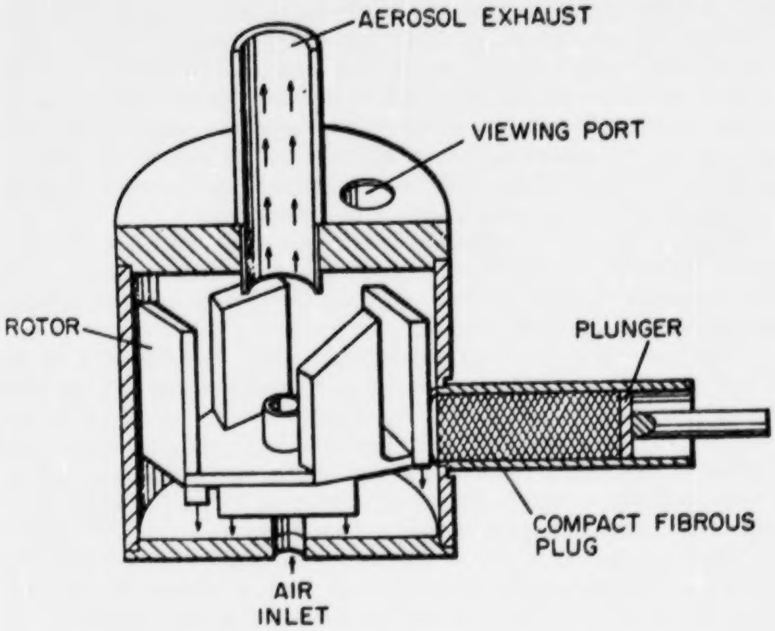

Figure 4.22. Schematic view of Ettinger's modification of Timbrell's fibrous aerosol generator. (Source: Adapted from Tillery et al. 1976. Used with permission of the publisher.)

Depending on the design, these can produce either liquid droplet or solid particle aerosols.

One type of liquid aerosol generator designed from some of the same principles used in dust dispensers is based on the aspirator. Basically the aspirator functions by creating a pressure drop across an orifice or capillary tube, which draws the liquid test agent up the tube where it is sheared into particles by the air stream. Although it is used with dusts, the aspirator can function more efficiently with liquids because the flow characteristics are more favorable and easily achieved. Thus, many types of liquid aerosol generators use the aspirator to atomize the liquid into particles.

A typical atomizer based on the above principle is the Laskin atomizer (Fraser et al. 1959). Pressurized air is pumped up a central, hollow tube and out four radially drilled holes at the top. Directly beneath these holes is a collar with four vertical capillary tubes lined up with the radial holes. As air passes over the capillaries, liquid is drawn up and broken into droplets. The test agent feed rate in this system is dependent on the venturi effect at the top of the capillaries and the air flow rate through the atomizer. Because the most efficient atomization of liquids occurs near the maximum air flow rate (Gage 1953), only a limited range of output variation is possible by changing the air flow rate. A motor-driven syringe delivery system for the test liquid can be used to avoid this limiting factor (Gage 1953). Although this system is used primarily with liquids, solid particles can be created by mixing in dry or hot air with the droplets to evaporate the liquid (Gage 1968), leaving crystals (when using a solution) or solid particles (when using a solid-liquid suspension).

A special category of aspiration atomizers is often referred to as nebulizers. Nebulizers atomize liquids, suspensions, and solutions, usually with compressed air to form particles, and also include size-selective devices, most frequently impaction surfaces.

The Vaponephrin nebulizer is an all-glass design consisting of a nozzle directing a stream of air across the top of a capillary tube, with the bottom of the tube resting in the test agent liquid. Directly opposite from the nozzle is a glass sphere that alters the direction of the air stream. This change in direction impacts larger particles on the sphere and allows smaller particles to remain airborne (Fraser et al. 1959). The whole complex is contained in a glass sphere to control loss of liquid. The critical dimensions of the design are the diameter of the nozzle orifice, which limits the air volume and determines the air pressure needed; the distance from the nozzle to the capillary; and the distance from the capillary to the impaction sphere.

Another simple nebulizer design (Figure 4.23) is the DeVilbiss nebulizer. This is also a glass design with a vertical aspirator (Raabe 1976). The impaction surface is the curved area of the exhaust tube, which restrains about 99% of the particles produced (Mercer et al. 1968b). Other impaction surfaces include: a series of baffle holes that require the particles to follow a tortuous path (the Dautrebande nebulizer), the curved walls of the generator (the Lauterbach nebulizer), a hollow cylinder around the aspirator jet (the Collison nebulizer), and a sharp right angle with a flat surface or a rounded curve baffle (Wright, Retec, and Lovelace nebulizers) (Whitby et al. 1965, Mercer et al. 1968, Mercer 1973, Raabe 1976). Some of the output characteristics of the common nebulizers are given in Table 4.8 (Tillery et al. 1976). In general, the differences in output characteristics are due to the internal geometry of the chambers and the flow rates through them (Tillery et al. 1976). For specific applications, these factors, and the critical dimensions mentioned above, can be modified to produce the desired output concentrations and particle sizes.

A unique variation of the nebulizer design is fractionation of the liquid stream by ultrasonic vibrations rather than by air pressure. Piezoelectric crystals are typically used to create the vibrations, which are transmitted through a coupling fluid (Figure 4.24) to the test liquid (Mercer 1973, Tillery et al. 1976). The test liquid reacts to the vibrations by forming a fountain above the crystal with droplets breaking off at the top. The size

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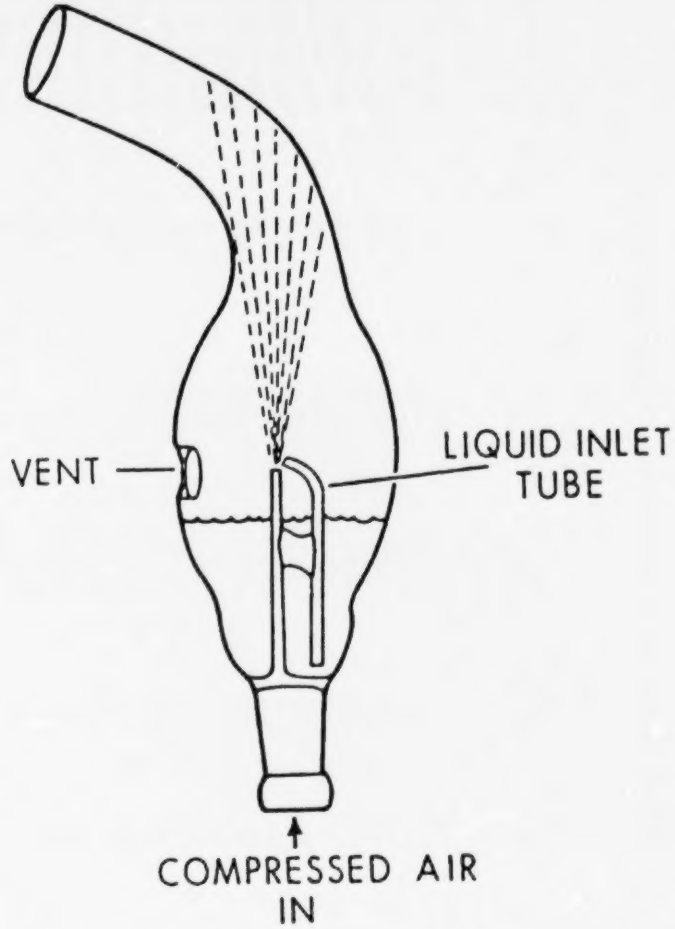
VENT
LIQUID INLET TUBE
COMPRESSED AIR IN

Figure 4.23. Diagram of the DeVilbiss nebulizer. (Source: Adapted from Mercer et al. 1968b. Used with permission of the publisher.)

Table 4.8. Output Characteristics of Some Compressed Air Nebulizers

Nebulizer	Jet pressure (psi)	Mass median diameter (μm)	Geometric standard deviation	Specific output (μL solution/L jet air)	Output (μL solution/min[a])	Reference
Vaponefrin	12	5.6	1.80	~29	117	Mercer et al. 1965
DeVilbiss #40	10	4.1	~1.85	16.0[b]	155	Mercer et al. 1968b
DeVilbiss #40	20	3.2	~1.85	13.8[b]	229	Mercer et al. 1968b
DeVilbiss #40	30	2.8	~1.85	12.8[b]	270	Mercer et al. 1968b
Bennett Twin (2814)	7.5	6.8	1.80	23.8	119	Mercer et al. 1965
Puritan (R6-051)	23	6.5	1.90	26.6	266	Mercer et al. 1965
Lauterbach	10	3.8	~2.05	3.9	30[c]	Mercer et al. 1968b
Lauterbach	20	2.4	~2.05	5.7	67[c]	Mercer et al. 1968b
Lauterbach	30	2.4	~2.05	6.0	91	Mercer et al. 1968b
Dautrebande D-30	10	1.7	~1.65	1.42	21	Mercer et al. 1968b
Dautrebande D-30	20	1.4	~1.65	2.3	49	Mercer et al. 1968b
Dautrebande D-30	30	1.3	~1.65	2.4	65	Mercer et al. 1968b
Lovelace	10	NG[d]	NG	15.3	14[e]	Mercer et al. 1968b
Lovelace	20	5.4	1.90	30	39[e]	Mercer et al. 1968b
Lovelace	30	NG	NG	35	58.3[e]	Mercer et al. 1968b
Collison	15	NG	NG	8.7	53	May 1973

Table 4.8. (continued)

Nebulizer	Jet pressure (psi)	Mass median diameter (μm)	Geometric standard deviation	Specific output (μL solution/L jet air)	Output (μL solution/min[a])	Reference
Collison	25	1.9	2.5	6.7	55	May 1973
Collison	30	NG	NG	5.8	55	May 1973
Retec	20	5.7	1.8	35.2	208	Raabe 1972
Retec	30	3.6	2.0	35.9	284	Raabe 1972
Retec	50	3.2	2.2	31.9	376	Raabe 1972

[a] Dioctyl sebacate is the nebulized liquid for the Collison, while water containing salt or a fluorescent dye is the nebulized liquid for the other nebulizers.

[b] Zero auxiliary air flow.

[c] Orifice diameter, 0.032 in.

[d] NG = not given.

[e] Orifice diameter, 0.001 in.

Source: Adapted from Tillery et al. (1976).

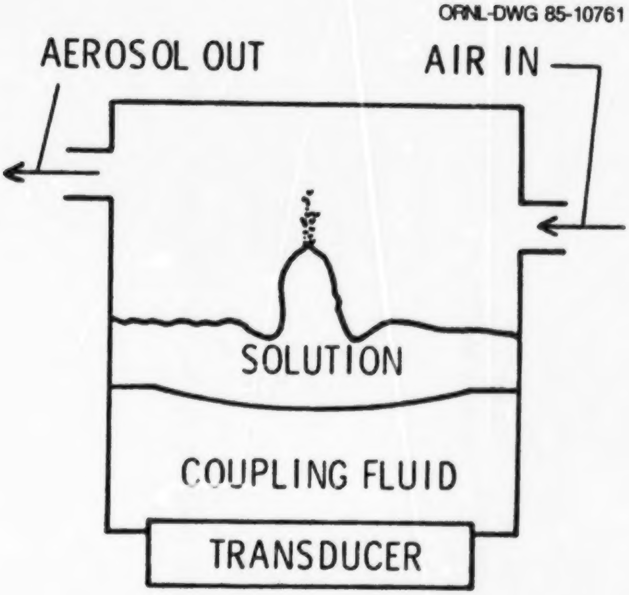

Figure 4.24. Schematic view of ultrasonic nebulizer. (Source: Adapted from Tillery et al. 1976. Used with permission of the publisher.)

of the droplets depends on the frequency of the acoustic field, the properties of the test chemical, and the rate at which they are removed from the fountain (Raabe 1976). A stream of air is routed through the chamber to disperse the accumulating aerosol. Some output characteristics of various ultrasonic nebulizers are given in Table 4.9. In general, ultrasonic nebulizers produce more monodispersed aerosols than do typical compressed air nebulizers (Mercer 1973). Kreyling and Ferron (1983) describe an ultrasonic nebulizer design that produces aerosols from low concentrated particle solutions, can be optimized to produce specific particle sizes by varying air pressure and impaction plate angle, and can deliver a constant concentration for more than one hour. Another form of ultrasonic nebulizer (the Hartman Whistle) utilizes compressed air shot into a resonant chamber to produce sound waves of 25 kilocycles, which immediately disperse any introduced liquid into a fine aerosol (Fraser et al. 1959).

The most commonly used technique for generating liquid aerosols, especially monodisperse aerosols, is the spinning disk generator first developed by Walton and Prewett (1949). The generator consists of a flat horizontal disk surface, which is rotated at high speeds (up to 100,000 rpm) by a direct drive air or electric motor (Raabe 1976). The test liquid is fed onto the center of the disk from a fixed-height reservoir or a syringe pump and is spread out in a thin layer by centrifugal force (Figure 4.25).

Table 4.9. Output Characteristics of Some Ultrasonic Nebulizers

Nebulizer	Frequency (MHz)	MMD (μm)	CMD (σ_g)	0.34λ (μm)	(μm)	Output (mL solution/min)	Reference
Mist O_2 Gen, 50-mL cup[a]	1.40	6.5	1.4	3.9	3.7	1.64	Mercer et al. 1968a
Mist O_2 Gen, 10-mL cup[a]	1.40	6.5	1.5	4.0	3.7	0.88	Mercer et al. 1968a
DeVilbiss, gain setting 2[b]	1.35	5.7	1.5	3.5	3.8	2.25	Mercer et al. 1968a
DeVilbiss, gain setting 3[b]	1.35	6.9	1.6	3.5	3.8	3.83	Mercer et al. 1968a
DeVilbiss, gain setting 4[b]	1.35	6.9	1.6	3.5	3.8	6.16	Mercer et al. 1968a
Denton-Swartz	1.00	NG[c]	NG	5.2	4.6	~1.00	Denton and Swartz 1974
Denton-Swartz	3.00	NG	NG				

[a] Mist O_2 Gen, model EN140, is available with two generation chambers: a 50-mL nebulizer cup with connections for an external reservoir and a 10-mL nebulizer cup.

[b] DeVilbiss, model 880, has four gain settings for power to crystal.

[c] NG = not given.

Source: Adapted from Tillery et al. (1976).

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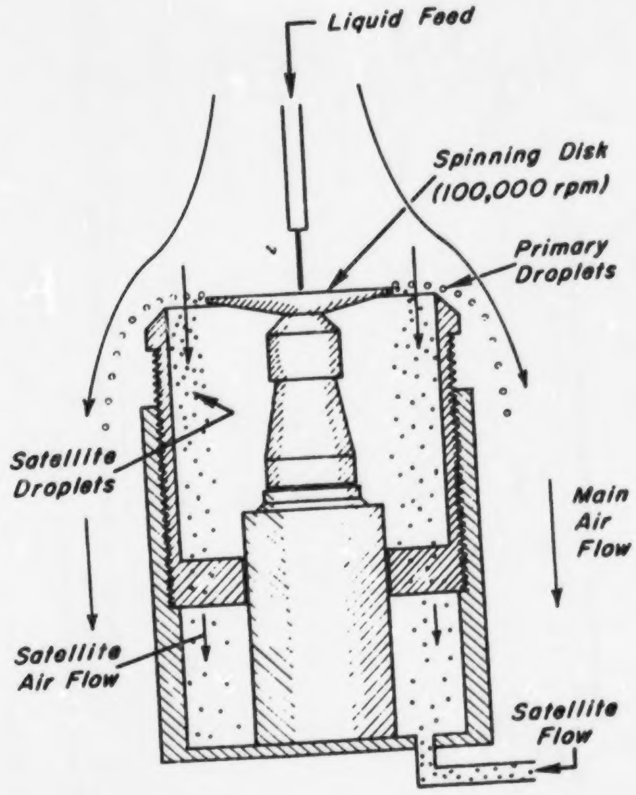
Liquid Feed
Spinning Disk (100,000 rpm)
Primary Droplets
Satellite Droplets
Main Air Flow
Satellite Air Flow
Satellite Flow

Figure 4.25. Schematic drawing of a spinning disk generator used to produce monodisperse aerosols of both soluble and insoluble forms from solutions of suspensions. Air flow into the satellite collector is adjusted so that the inertia of the primary particles allows them to enter the main air flow. (Source: Adapted from Raabe 1976. Used with permission of Academic Press.)

As the liquid layer reaches and accumulates at the edge of the disk, droplets are thrown off when the centrifugal force exceeds the restraining capillary action (Mercer 1973). The majority of the liquid mass forms particles of the desired size, but smaller satellite droplets are also formed, usually four for every primary droplet (May 1949). A stream of air, insufficient to overcome the inertia of the primary droplets, is used to control the satellite droplets.

A similar generator, the spinning top, also was designed by Walton and Prewett (1949) and modified by May (1949, 1966). It used a flat disk with a conical bottom cut into turbine blades. Pressurized air directed at the blades provided support and rotation for the disk. Generally, spinning tops are smaller and operate at higher speeds (up to 240,000 rpm) than the spinning disk (Mercer 1973, Tillery et al. 1976). Using the spinning top, May was able to generate monodisperse particles with coefficients of variation as low as 0.05 when the disk surface was properly treated. The May spinning top generator is usually mounted within the exposure chamber, as droplets are thrown off in a 360° area around the top. A recent modification incorporates an encapsulating plenum to control the aerosol generated, allowing it to be used outside the chamber (Gussman 1981).

The droplets created by spinning disks and tops are mostly monodisperse when the liquid feed rate is slow with particle size varying with the rotational speed (Roe 1968). Table 4.10 shows the change in particle size with the rotational speed of a spinning disk that used an air-driven tool grinder motor (Whitby et al. 1965). The range of particle sizes possible with the spinning disks is usually above 0.5 μm and can be up to 50 μm (Raabe 1976). Dry particles can also be created with the spinning disk by evaporation of the liquid solvent. Hurford (1980) describes a modification of the May generator that produces a monodisperse, solid aerosol with a size range of 1 to 6 microns and a standard deviation of 1.1. The sizes were verified by optical and scanning electron microscopy. The modification to the May generator, by Gussman (1981), also provides the smooth, nonturbulent column of rising air needed to evaporate the liquid solvent and produce particles of 5 μm or less in diameter.

The condensation of vapors on nuclei has also been used to create monodisperse aerosols. Sinclair and LaMer (1949) used the apparatus shown in Figure 4.26 to form particles with a narrow size range. The liquid test agent is first vaporized in the boiler, then nuclei (from an electric spark or NaCl–coated wire source) are added, and the mixture is carried by a stream of air to the reheater. Because the reheater is at a higher temperature, complete vaporization of the liquid is assured. The mixture then enters the chimmey, where it is cooled to allow condensation of the vapor on the nuceli. By carefully controlling the nucleus size and the cooling process, particles of uniform size are created (Mercer 1973, Tilley et al. 1976). Modifications in the basic design were made by Rapaport and Weinstock (1955), Muir (1965), and Liu et al. (1966). These modified

Table 4.10. Operating Conditions at Which Model I Spinning Disk Was Used[a]

Operating conditions	Air motor speed (rev/min)				
	70,000	45,000	30,000	20,000	12,000
Main droplet diameter (μm ± 10%)	19	28	41	60	100
Dye concentration by weight (g/g)	5×10^{-5} to 3×10^{-3}	1×10^{-5} to 3×10^{-3}	5×10^{-5} to 3×10^{-3}	5×10^{-5} to 3×10^{-3}	5×10^{-5} to 3×10^{-3}
Solid particle diameter (μm)	0.6-2.4	0.6-3.5	1.3-5.1	1.9-7.5	3.2-12
Discharge air flow (cfm)	100	100	70	50	40-50
Orifice diameter (in.)	6	6	7.5	9	15
Satellite air flow (cfm)	15	15	15	16	18
Satellite funnel diameter (in.)	2.5	3	4	5	6
Liquid feed rate (mL/min ± 20%)	2.5	3	3	3½	4
Feed needle size (number)	22	22	22	19	19
Particle generation rate (particles/min ± 20%)	350×10^5	130×10^6	40×10^6	15×10^6	3×10^6
Projection of the hypodermic needle below the satellite funnel (in.)	1/16	3/32	1/8	3/16	3/16

[a] Data obtained using methylene blue dye in alcohol. Disk diameter = 4.65 cm.

Source: Adapted from Whitby et al. (1965).

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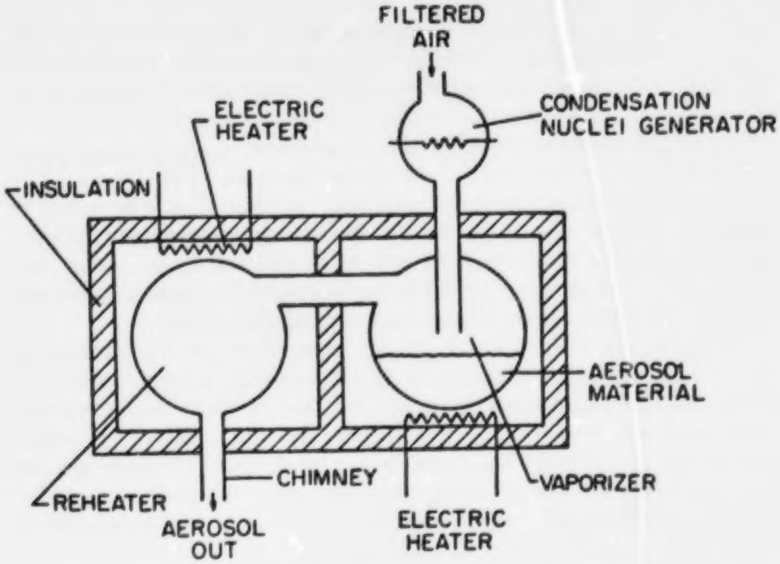
FILTERED
AIR
CONDENSATION
NUCLEI GENERATOR
ELECTRIC
HEATER
INSULATION
AEROSOL
MATERIAL
CHIMNEY
VAPORIZER
REHEATER
AEROSOL
OUT
ELECTRIC
HEATER

Figure 4.26. Schematic view of Sinclair-LaMer monodisperse aerosol generator. (Source: Adapted from Tillery et al. 1976. Used with permission of the publisher.)

designs are capable of producing monodisperse aerosols in the range of 0.3 to 1.4 μm (Mercer 1973, Tillery et al. 1976).

4.5. CONCLUSIONS AND RESEARCH RECOMMENDATIONS

4.5.1 Conclusions

Toxicity tests designed to evaluate effects from inhalation exposure involve many of the same variables as tests using other exposure routes. Exceptions to the standard protocols include: (1) selection of test species should consider anatomical and physiological similarity of respiratory tract to that of humans; (2) exposure modes can be intermittent (6 to 8 h/day for 5 days/week) or continuous; (3) determination of actual effective dose is more complicated—generally the chamber concentration times length of exposure is used; and (4) observations for effects include more extensive pathological examination of respiratory structures and use of respiratory function tests.

An important aspect of inhalation testing is the design of the test chamber, of equipment for generating the exposure atmosphere, and of equipment to monitor chamber atmospheric conditions and test material concentrations. In most tests, operation of the test chamber is in a dynamic mode, where a continuous stream of test agent and air is vented through the chamber. The test chamber is usually designed for whole-body exposure, although nose- and head-only exposure systems are also used. The shape of most large chambers is cubical or hexagonal with pyramidal or conical additions at top and bottom to increase distribution efficiency of air within the chamber. Chamber size is governed by air distribution and animal volume considerations. Construction materials need to be smooth-surfaced, nonabsorbant materials that are resistant to a wide range of chemicals. Airfow systems for the chamber should contain units to condition, filter, meter, and control the flow of air and test agent to and from the chamber. The chamber should be maintained at a negative operational pressure and within narrow temperature and humidity ranges.

Sampling and monitoring of the test atmosphere and chamber conditions should be performed during operation. Sampling techniques for monitoring gas and vapor atmospheres usually rely on filters and automated analytical procedures. Aerosol atmospheres are more difficult to sample, particularly at the breathing zones, and a whole array of equipment has been developed for this, including elutriators, cascade impactors, centrifugal force samplers, and optical monitors. Some type of size separation for the particles is usually part of the procedure.

Generation of the test atmospheres also differs based on the type of test material. Gas and vapor generation is usually achieved by release into the airstream from high pressure cylinders or through vaporization using controlled heating or counter-current techniques. Aerosol atmospheres can be generated in monodisperse or polydisperse forms. Generation of aerosols from solid materials can be accomplished from loose materials by use of aspirators and a vibrating, rotating, or stirring technique to create a source suspension. Compacted forms of test materials can be put into an aerosol suspension by abrading the surface with a scraper blade in the path of an airstream. Generating aerosols from liquid test materials can be done in more varied methods. Aspirators with an impaction surface can produce particles of limited size ranges. This technique can be combined with a fractionating device to produce aerosols. Applying the liquid to a spinning disk or top is another technique frequently used to produce liquid aerosols.

4.5.2 Research Recommendations

More research is needed on:

- Applicability of test species, particularly regarding metabolic similarity and particle deposition sites in the respiratory tract.
- Determination of actual effective doses in inhalation testing. This could include actual dose delivered to animal lungs, dose delivered to blood of test animals, and dose reaching target organs.

- The use of alternative testing methods for inhalation toxicity, perhaps more in vivo tests such as lavage fluid tests or in vitro tests that could be used to determine acute or target organ (lung) effects.
- The use of respiratory function tests to widen their applicability, increase knowledge as to the significance of their data, and provide data for comparisons between test animals and humans.
- The various types of atmosphere generating equipment, particularly aerosol generators, including comparisons of efficiency, ability to maintain desired particle size and distribution ranges, and avoidance of operating problems (e.g., particle clumping on size separators in aerosol generators).
- The potential for extrapolation of results from oral or intravenous exposure studies to inhalation studies for non–respiratory-tract effects or for certain chemical classes. This might allow reduction in the total number of inhalation studies or in those studies aimed at systemic effects of inhalation exposure.
- The extrapolation of inhalation toxicity data gained from animal studies to human exposure. Although this problem is not specific to inhalation studies, it is one of the most important areas in which existing data are insufficient to determine the true relationships between animal and human studies.
- The relationship between toxicity evaluation of a chemical and hazard evaluation of a chemical, particularly as applied to inhalation studies. The regulatory guidelines require toxicity data applicable to general situations, but the real life situation requires hazard (toxicity × exposure situation) data for determining the impact of a chemical in a specific situation. How to satisfy both requirements without duplication of testing efforts needs more study.

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16. ABSTRACT

This document is the second of a two-part literature analysis of parameters associated with the various toxicity testing methods (test animal selection, pathology, etc.). Acute, subchronic, chronic, and carcinogenic testing methods are covered in ORNL/EIS-151. Testing methods for developmental toxicity, immunotoxicology, and inhalation toxicology and research needs associated with these areas are covered in this volume, ORNL-6094. These reports were prepared for the purpose of assisting and supporting the U.S. Environmental Protection Agency in its efforts to develop guidelines for more efficient and economical testing procedures.

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